

DEVELOPMENT OF THE ADOLESCENT PREFRONTAL CORTEX AND BASOLATERAL  
AMYGDALA AND THE EFFECTS OF PUBERTY AND ALCOHOL EXPOSURE

BY

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DISSERTATION

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## **ABSTRACT**

Human structural magnetic resonance imaging (MRI) studies indicate that some neural regions such as the prefrontal cortex (PFC) and the amygdala continue to develop throughout adolescence into early adulthood. These studies have specifically shown that the volume of the PFC increases until puberty and then decreases until early adulthood. By contrast, the amygdala has been shown to increase in volume through the adolescent period. Using the rat as a model of these changes, our laboratory has previously found that both of these structures show a decrease in the number of neurons between postnatal day (P) 35 and P90, the equivalent adolescent period in the rat (Markham et al, 2007; Rubinow and Juraska, 2009). The studies described here were designed to further elucidate neuroanatomical changes in the brain during the adolescent period as well as investigate how intrinsic and extrinsic factors may disrupt this development. In chapter 2, adolescents and adults were compared in a behavioral task dependent on the medial prefrontal cortex (mPFC). Adolescents were able to perform the task. On certain delays (15s and 30s), however, they were consistently worse than adults indicating that behavior that depends on the mPFC is not yet at adult levels. To identify further neuroanatomical changes in the mPFC and the basolateral amygdala (BLA), dendritic arborizations and dendritic spines were analyzed in chapter 3. These studies indicated a growth of dendrites and dendritic spines between P20 and P35 (prepuberty) in both sexes as well as both structures. After P35, the dendrites of the mPFC were shown to prune predominately in females, whereas in the BLA, dendrites slightly increased in length but significantly increased in the number of branches in both sexes. During this period of neuroanatomical change, adolescent rats were exposed to alcohol in a binge-like manner and then sacrificed in adulthood to quantify the number of neurons and glia. Results reported in chapter 4 revealed no significant differences in the total number of neurons in the mPFC. However there was a significant decrease in glia that occurred in the male mPFC. No differences were found in any measure

in the BLA. In chapter 5, pubertal hormones were removed by ovariectomizing or castrating animals prior to puberty. Results showed that removal of the testes caused no effects in males but there were changes in the number of neurons and glia in females. It was found that females without ovaries during puberty had more neurons and a greater number of glia, thus eliminating sex differences. Taken together these experiments reveal further neuroanatomical changes in the mPFC and the BLA. Furthermore they highlight how pubertal hormones are involved in the adolescent development of the mPFC and the cellular effects of alcohol use within the adolescent period.

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## **Chapter 1**

### **Introduction**

The adolescence period has been defined as a transitional phase from childhood to adulthood (Spear, 2000). Neurobiologically, it is a time where some brain regions are fully developed, where others are not yet at adult levels. Human structural magnetic resonance imaging (MRI) studies have shown that neural regions that are involved in cognition continue to develop in to adolescence and early adulthood while most sensory areas are fully developed (Gogtay et al, 2004). The precise age range where adolescence occurs varies from individual to individual. A conservative estimate is 12-18 years of age in humans and P28 to P42 in rodents (Spear, 2000), but neuroanatomical changes have been shown to occur until 22 years of age in the human and P60 in the rat. Moreover, the onset of puberty is defined as the beginning of the adolescence period and puberty in male rodents does not begin until around P42-P45 and the onset of menses in humans is now age 11. Therefore a better estimate should be 11-22 in humans and P28-P60 in rodents. Neuroanatomical changes in volume and neuron number have been shown to be controlled by pubertal hormones in reproductive areas (Schulz et al, 2009) and nonreproductive areas (Nunez et al, 2001). Using the rat as a model, the four experiments in the following chapters investigated adolescent neural development in two brain regions: the medial prefrontal cortex (mPFC) and the basolateral nucleus of the amygdala (BLN). These areas are highly interconnected with each other (Van Eden and Uylings, 1985; Amaral, 1986) and both have been shown to be involved in cognition (Pare, 2003; LeDoux, 2003; Uylings et al, 2003; Dias and Aggleton, 2000; Dunnett et al, 1999). Also these brain areas have shown to develop late in humans and rats (Giedd et al, 1996a; Giedd et al, 1996b; Sowell et al, 1999; Jernigan; et al, 1991; Markham et al, 2007; Rubinow and Juraska, 2009). Prior work in our laboratory has found decreases in the number of neurons in both of these structures across adolescence (Markham et al, 2007; Rubinow and Juraska, 2009). The following set of studies focused on behavioral

consequences of late development and describes dendritic alterations that are occurring over adolescence. Additionally, because the time of adolescence is often where individuals are first exposed to alcohol, chapter 4 examined how alcohol exposure may perturb normal neural development during adolescence. Moreover, it has previously been found that the sex difference in the visual cortex is caused by a decrease in neurons triggered by the release of ovarian hormones at puberty (Nunez et al, 2002). In the last experiment we tested whether the same mechanism is causing the decrease in neurons in the mPFC.

### **Adolescent Behavior**

Adolescence is a period of multiple psychosocial changes that include: a shift of social behaviors from parents to peers, heightened emotional responses, as well as increased risk-taking and novelty-seeking behaviors (Arnett, 1999; Spear, 2000). These behaviors aid the individual to obtain autonomy from parents, establish independence in their environment and optimize reproduction (Spear, 2000; Ernst and Fudge, 2010). However, in humans, these behaviors can have negative consequences such as illicit drug use, drug addiction or lethal accidents (Spear, 2000; Ernst and Fudge, 2010; Steinberg, 2004). Moreover, affective and psychotic mental disorders first appear during this period (Arnett, 1999; Adriani&Laviola, 2004; Rutter et al, 2003; see Ernst and Fudge, 2010). These behavioral changes have been associated with the both structures investigated in this manuscript. In fact, interactions between the prefrontal cortex (PFC) and the amygdala have been found to play a role in emotional regulation, the regulation of fear responses, and avoid/approach behavior (for review see Ernst and Fudge, 2010; Killgore et al, 2001; Monk et al, 2003).



In adolescence, cognitive behaviors are not yet at adult levels. This includes poorer working memory, attention, and slower processing speeds in many human tests of cognition (for reviews see Graber et al, 1996; Keating, 1990; Anderson et al, 2001). For instance, in humans, performance on working memory tests, such as the digit span and attentional-shift working memory components of the contingency naming task, improve across adolescence (Anderson et al, 2001). Both of these tasks involve working memory and attentional control. Adolescents have also shown deficits in planning skills and goal setting behaviors within tasks like the Tower of London and the complex figure of Rey test (Levin, 1991; Anderson et al, 2001). All of these cognitive processes involve the PFC (Benton, 1968; Fuster, 1993) which, as previously mentioned, is not fully neuroanatomically mature until after adolescence (Giedd et al, 1996a, 1996b, Jernigan et al, 1991). Overall processing speed also improves during the adolescent period (Anderson et al, 2001) which may be due to the lack of myelination in early adolescence (Yakovlev and Lecours, 1967).

Adolescent behaviors in rodents are quite similar to humans. Rats exhibit increases in social interaction, play, and locomotor activity as well as increased novelty-seeking behavior (Primus and Kellogg, 1991; Spear et al, 1980; Spear, 2000). Tasks involving spatial memory, emotional components of learning and memory and fear responses are not yet at adult levels in adolescent rats (Niemi and Thompson, 1980; Wiedenmayer, 2009; Spear, 2000; Schenk, 1985; Rubinow et al, 2009a). However, there are no studies comparing adolescents and adults on PFC-dependent tasks in rats. Therefore, Chapter 1 compared adolescent performance on a PFC-dependent task to adult rats.

## **Adolescent Neural Development of the PFC and the Amygdala**

The PFC has been of interest due to multiple human imaging studies documenting gray matter volume changes occurring well into the 2<sup>nd</sup> decade of life (Giedd et al, 1996b; Jernigan et al, 1991; Sowell et al, 1999). Moreover, this brain structure and its connections with subcortical areas like the amygdala and hippocampus, make it heavily involved in emotional responses, as well as attentional and impulse control that may lead to unwanted behavioral consequences (i.e. drug use) within the adolescent period. In humans, structural MRI has demonstrated PFC gray matter volume changes in an 'inverted U' shape pattern where there are increases throughout childhood until 11-12 years of age, then volume peaks and decreases before stabilizing around 20 years of age (Giedd et al, 1996b; Jernigan et al, 1991; Sowell et al, 1999). The underlying mechanisms of these changes may be reductions in synapses or dendritic material, as well as cell death. All of these mechanisms have been known to be important actions in prenatal as well as early postnatal development in order to establish proper neural connections.

Apoptosis, or programmed cell death, in postmitotic neurons occur postnatally in humans and rats (Brown et al, 1991, Stiles, 2008). In rats, cell death has been well documented to occur within the first 2 postnatal weeks in the cortex (Ferrer et al, 1992), but one study, in the visual cortex, has observed cell death in rats as late as P25 (Nunez et al, 2001). Moreover, studies reporting decreases in the total number of neurons between P35 and P90 rats in the mPFC and the BLN (Markham et al, 2007; Rubinow and Juraska, 2009) provide evidence of cell death occurring in the adolescent period.

Unlike the PFC, imaging studies examining the entire amygdala gray matter volumes find an increase across adolescence (Giedd et al, 1996a; Merke et al, 2003; Schumann et al, 2004). This seems to be inconsistent with the findings in the rat that reported decreases in the

number of neurons in the BLN (Rubinow and Juraska, 2009). However, volume changes did not accompany the cell loss. The discrepancy in the results between human and rat data is most likely due to the spatial limitations of MRI. The amygdala is made up of several nuclei and unfortunately all the nuclei have been named differently throughout the years. For simplicity, this manuscript will describe the different nuclei using the names from those that have investigated the behavioral roles of the amygdala (Azad et al, 2004; Koo et al 2004; Paré et al, 2003, Scott and Shinnick-Gallagher, 2005). This describes 3 major nuclei of the amygdala: the basolateral complex, the medial amygdala, and the central amygdala. The basolateral complex (BLA) is the entire teardrop structure in which the amygdala received its name. The BLA contains two major nuclei: the lateral nucleus (LA) and the basolateral nucleus (BLN). The medial and central amygdala are nuclei situated just medial to the BLN complex in the rat and have been anatomically described to resemble neurons of the striatum whereas the BLN and LA neurons are more cortical-like (Sah, 2003; McDonald, 1998). The medial amygdala plays a large role in sexual behavior; the central amygdala is involved with autonomic responses to fear via its connections with brain stem nuclei (Sah et al, 2003); whereas the BLN is involved in learning and memory, regulating fear responses, and approach/avoidance responses (Ernst and Fudge, 2010). Furthermore, the medial amygdala has also been found to change across adolescence. Work by the laboratory of Cheryl Sisk has shown increases in the volume of the medial amygdala across adolescence as a result of increased cell soma size (Romeo and Sisk, 2001) and increased number of neurons (Ahmed et al, 2008). Due to low resolution capabilities, MRI does not have a high enough resolution to parcellate individual nuclei of the amygdala; therefore the increase in this nucleus could be the increase that MRI studies are reporting within the amygdala.

All of the work demonstrated in this dissertation investigated the nuclei contained in the BLN only. The BLN is the most connected nucleus to the mPFC (Amaral, 1986). Furthermore, our laboratory has demonstrated cellular losses, in both neurons and glia, in this particular nucleus across adolescence (Rubinow and Juraska, 2009). However, unexpectedly it was found that the BLN volume did not decrease with the decline in cell numbers. Therefore, the study in chapter 3 measured dendritic alterations during the adolescent period that may be expanding to replace the volume that neurons and glia once occupied.

It is well established that the nervous system first overproduces synapses throughout development and then eliminates unnecessary contacts (Brown et al 1991; Stiles, 2008). Several areas of the cortex prune synapses into late childhood and adolescence (Bourgeois and Rakic, 1993; Bourgeois et al, 1994; Woo et al, 1997; Yildirim et al, 2008 ; Meyer et al, 1978), but the PFC is one of the latest with the number of synapses decreasing until age 16 in humans (Huttenlocher, 1979; Huttenlocher and Dabholkar, 1997). This pattern is comparable to the volume measures in gray matter across the cortex in structural MRI studies, where sensory cortical areas appear to finish developing before more cognitive cortical areas, like the prefrontal cortex (Gogtay et al, 2004). Studies examining the arborization of dendritic trees in human postmortem tissue have found that in adulthood the sensory areas of the brain possess less complex dendritic trees than cognitive areas of the brain (Jacobs, 1997), but early in development the sensory areas have more elaborate trees (Travis et al, 2005) indicating that dendritic arborization is developing later in cognitive areas (Stiles, 2008). In the visual cortex, dendritic trees of pyramidal neurons are adult-like in form and extent by day 15 in the rat (Juraska and Fifkova, 1979), but some development occurs after day 15. In adolescence the visual cortex peaks in dendritic spine density and then decreases (Juraska, 1982; Boothe et al,

1979), similar to other cortical areas (Yildirim et al, 2008; Meyer et al, 1978; Wise et al, 1979) and the medial amygdala (Zehr et al, 2006). Since dendritic spine density is an indication of the number of excitatory synapses this demonstrates synaptic remodeling in all of these areas during the adolescent period (Harris, 1999). Dendritic growth and retractions have also been observed in the visual cortex, which also provides more evidence for the refinement of synapses in the visual cortex during adolescence (Juraska, 1982). Relatively little is known about the dendritic arborization and spine changes occurring during adolescence in the mPFC. One study has reported adolescent changes in layer 3 pyramidal neurons of the mPFC (Markham et al, 2012). It found dendritic growth and some pruning of spines in these cells during the adolescent period. In chapter 3, we also investigated dendritic arborization and dendritic spines but in layer 5 pyramidal neurons. This specific layer is the primary output layer of the cortex and has shown to be more sexually dimorphic than layer 3 in neuron loss measures (Markham et al, 2007).

Some studies have shown alterations in neurogenesis or gliogenesis in adolescence. Two areas of the brain that continue to create new neurons are the dentate gyrus of the hippocampus and the subventricular zone, an area where cells arise and later migrate to the olfactory bulb (Kempermann, 2011). In the adult neocortex and the medial amygdala, very low levels of neurogenesis have been reported (Cameron and Dayer, 2008; Ahmed et al, 2008). However, in adolescence there is no evidence of large amounts of neurogenesis in the PFC or the BLN where prior studies report decreases in neurons only (Markham et al, 2007; Rubinow and Juraska, 2009). Unlike neurons, glia cells, such as microglia, astrocytes and oligodendrocytes, continue to proliferate throughout the life span of humans and rodents in all regions of the brain (Lee et al, 2000; Ligon et al, 2006), but what their proliferation rates are is currently unknown. In the mPFC an increase in glia has been shown throughout the adolescent period in the mPFC in males,

but not in females (Markham et al, 2007). In the BLN a decrease was found in both sexes during adolescence (Rubinow and Juraska, 2009). Therefore glia proliferation and death appears to be both region and sex specific.

Alcohol use is prevalent during adolescence (Brown and Tapert, 2004) and currently it is not known how it may disrupt neural development. It is known that ethanol sharply increases naturally occurring neuronal death throughout the brain during the prenatal and early postnatal period in rats (Goodlett and Eilers, 1997; Ikonomidou et al., 2000; Miller and Potempa, 1990), including in the mPFC (Mihalick et al., 2001). In the BLN, although less studied, ethanol has been shown to permanently alter glutamatergic and GABAergic neurotransmission in 4-6 week old rats (Christian et al., 2012; Silberman et al., 2009). The effects of alcohol exposure on normally occurring neuron loss in the BLN and mPFC during adolescence were tested in Chapter 4.

### **Sex Differences and Puberty**

Puberty is defined as a process that falls within the adolescent period which involves physical and endocrine changes that allow sexual maturity to occur (Sisk and Zehr, 2005). Puberty occurs in girls around 12 years of age and in boys around 14; however there are reports of puberty occurring earlier (Aksglaede et al, 2008). During this time hypothalamic neurons induce the release of pituitary hormones, which starts the release of ovarian hormones in females and signals for further increases of testosterone and spermatogenesis in males. Additionally, these hormones cause permanent neuroanatomical changes in both males and females in both reproductive and nonreproductive regions of the brain (for review see Schulz et al, 2009).

In adolescent MRI studies sex differences have been reported within the mPFC (Giedd, 1999) indicating that gray matter volume peaks earlier in females (11.0 years) than males (12.1 years). Additionally, in the MRI imaging of the amygdala, gray matter volume increases mostly in males (Giedd, 1996b). However, that study had a very small sample size and, as mentioned above, could not differentiate between the very different nuclei within the amygdala. In the cellular studies performed in rats, no sex differences have been found in the BLN; however sex differences do occur in the visual cortex and the mPFC in adulthood (P90). These sex differences do not appear until after puberty (Reid and Juraska, 1992; Nunez et al, 2002; Markham et al, 2007). It is currently not known if the sex differences in the mPFC are dependent on gonadal steroids coming from the ovaries of the female or the androgens from the testes in males. As noted, the visual cortex has a similar sex difference where males have more neurons than females. In visual cortex, the removal of ovarian hormones in females eliminated the sex difference in adulthood (Nunez et al, 2002). More evidence of female gonadal steroids promoting late cell death was demonstrated by a late 'wave' of cell death found just prior to puberty in the visual cortex of females, not males (Nunez et al, 2001). We infer that similar events in the mPFC are occurring. There is also research in brain regions that play a role in sexual reproduction that implicate androgens in sex differences occurring after puberty (De Lorme et al, 2012; Schulz et al, 2009). The experiment described in chapter 5 examined the effects of ovarian hormones and androgens in the adolescent development of the mPFC.

### **Aims of the Current Studies**

The set of studies within the following chapters aimed to expand the current knowledge about the adolescent mPFC and BLN in male and female rats. Behaviorally, the lack of maturity

in the BLN has shown to cause negative consequences in the performance of a BLN-dependent task (Rubinow et al, 2009a), but the same has not been confirmed for a PFC-dependent task. To accomplish this, the experiment in Chapter 2 compared adolescents and adults in the delayed spatial alternation T-maze, a PFC-dependent task, which is similar to working memory tasks in humans where adolescents did not perform as well.

As outlined above, previous data from our laboratory has demonstrated both of these structures lose neurons during adolescence; however if this cellular loss effects dendritic arborization and dendritic spines has yet to be determined. This was examined in Chapter 3. From volume estimates indicating decreases in the mPFC during this time, we predict to observe pruning in the mPFC, but because we did not find any volume changes in the BLN after neuron and glia decreases, we predict increases in dendritic branching and lengths. Additionally, substances, such as alcohol, can disrupt the cellular changes that occur in adolescence which is important because alcohol use primarily begins in the adolescent years when these structures are still developing. Therefore the effects of alcohol were investigated in Chapter 4. Moreover because we do not know what the role pubertal hormones play in the cellular loss and sex difference in the mPFC, we removed the influence of pubertal hormones by ovariectomy or castration before puberty and compared the anatomy of gonadectomized and intact animals in Chapter 5. Therefore this set of four studies was designed to build upon previous data from our laboratory about adolescent brain development and to investigate intrinsic and extrinsic factors that may affect it.



## **Chapter 2**

### **Adolescent Performance on the Delayed Spatial Alternation**

#### **T-maze<sup>1</sup>**

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<sup>1</sup> Chapter 2 is an adaptation from a previously published paper entitled “Delayed alternation in adolescent and adult male and female rats” by Koss, W.A, Franklin, A.D., and Juraska, J.M (2011).

## Introduction

The prefrontal cortex (PFC) is an area of the brain that is involved in many executive functions such as attention, working memory, and goal-oriented behavior. In humans, adolescent performance on these tasks is not yet at adult levels (Anderson et al, 2001; Levin et al, 1991). Moreover, many studies have documented the continuing maturation of the human prefrontal cortex throughout adolescence. Magnetic resonance imaging (MRI) and postmortem studies have found increases in white matter (Giedd et al, 1996b; Paus, 2005; Pfefferbaum et al, 1994; Sowell et al, 1999) and decreases in frontal gray matter volume throughout adolescence into young adulthood (Giedd et al, 1996b; Jernigan et al, 1991; Sowell et al, 1999). Underlying causes of gray matter volume decreases could be dendritic reductions, axon withdrawal, cell death, and/or synaptic pruning. In humans and non-human primates, losses of synapses have been reported (Anderson et al, 1995; Bourgeois et al 1994; Huttenlocher, 1979). Functional consequences of these changes may be increased cognitive speed and capacity in PFC-dependent tasks. Interestingly, decreases in gray matter volume have been correlated with increased performance in PFC-dependent behavior (Casey et al, 1997).

Studies using rodent models have also found that the development of the PFC is still occurring in adolescence. The medial prefrontal cortex (mPFC) of the rodent is analogous to the human PFC and the rodent mPFC is divided into the dorsal anterior cingulate (ACd), the ventral anterior cingulate (ACv), the prelimbic area (PL), and the infralimbic area (IL) (Uylings et al, 2003; Vertes, 2006). Recent research in adolescent rats has paralleled human imaging studies, reporting significant decreases in the volume of the ventral mPFC (PL and IL) as well as an increase of frontal white matter occurring from postnatal day 35 (P35; puberty) to P90 (Markham et al, 2007). Additionally, a significant decrease in neuron and glia number was found providing

a cellular basis for the volume decrease (Markham et al, 2007). Other related changes in connectivity have been found. Axons originating from the basolateral amygdala (BLN) are continuing to branch in the rat adolescent mPFC (Cunningham et al, 2002) and conversely axons from the mPFC to the BLN are being pruned from late adolescence to adulthood (Cressman et al, 2010). Interestingly the BLN is another brain region that has a decrease in neurons between adolescence and adulthood (P35 and P90; Rubinow and Juraska, 2009) similar to the mPFC.

Neurotransmitter changes have also been documented in adolescent rats. NMDA receptors peak at P28 and then decline by age P60 (Insel et al, 1990). Inhibitory GABA<sub>A</sub> receptors decrease in sensitivity (Kellogg et al, 1993) whereas the GABA/B<sub>DP</sub> receptor complex increases in sensitivity (Kellogg et al, 1993; Primus and Kellogg, 1991). Additionally, both dopamine and cholinergic innervation increase in the mPFC, accompanied by pruning of dopaminergic receptors throughout adolescence (Gould et al, 1991; Kalsbeek et al, 1988; Andersen et al, 2000). Together the continuing development of neurotransmitter systems and the changes in structural neuroanatomy undoubtedly have large impacts on behavior and cognition.

Behaviorally, rats show behavioral changes during the adolescent period much like humans. For example, adolescent rodents display increased social interaction (Primus and Kellogg, 1991) and risk taking behavior (Laviola et al, 2003; Spear et al, 1980; Stansfield and Kirstein, 2006). However, cognitive abilities have rarely been studied in adolescent rats, and the results have been inconsistent. Adolescents have shown deficits on the water maze and on a complex avoidance task (Schenk, 1985; Niemi and Thompson, 1980) but not on a simple avoidance task or the radial arm maze (Bauer, 1980; Chambers et al, 1996). It has been suggested that this is because many tests of cognition in rodents rely on activity and exploration which are naturally elevated in the adolescent rat (Spear and Brake, 1983).

Contributing to these neural and behavioral changes is the presence of hormones associated with puberty. Pubertal hormones play a role in forming sex differences in anatomy as well as some behavior (for reviews see McCormick and Mathews, 2007; Schulz et al, 2009). For instance sex differences in activity and maze learning do not appear until after puberty (Kanit et al, 2000; Krasnoff and Weston, 1976) and ovariectomy prior to puberty has been shown to attenuate sex differences in neuron number in the visual cortex (Nunez et al, 2002). Additionally, the timing of the decrease in gray matter in humans and the loss of neurons in the rat PFC, as well as the BLN, occur after puberty (Giedd et al, 1996b; Markham et al, 2007; Rubinow and Juraska, 2009). Moreover, prior work from our laboratory indicated a larger neuron loss in females (19%) than in males (5%) in the mPFC resulting in a sex difference in neuron number in adult rats that was not present in adolescents (Markham et al, 2007). Given this, the current study investigates possible sex differences in a mPFC-dependent task.

In the present experiment, performance on the delayed alternation task was tested in adolescent and adult rats of both sexes. The delayed alternation T-maze task has been shown to rely on the mPFC (Carter et al, 1995; Dias and Aggleton, 2000; Dunnett et al, 1999; Izaki et al, 2001). Freeman and Stanton (1992) and Watson et al (2009) have demonstrated that rats can acquire and perform this task before adolescence. However, it is not known how the performance of adolescent rats will compare to that of adult rats. Additionally, it is not clear whether there are sex differences in the task at either age. Both of these questions will be tested here for further understanding of functional consequences of the continuing maturity of the mPFC.

## **Methods**

### ***Subjects***

Long-Evans hooded rats were born in the Psychology department vivarium from parents that were obtained from Simonsen Labs (Gilroy, CA). Animals were weaned at day 23 from 6 different litters and housed 2-3 per cage in a 12:12 light/dark cycle where lights were on at 8am with water *ad libitum*. They began delayed alternation training at day 24 (adolescent group; male n=8 and female n=8) and day 80 (adult group; male n=8, female n=9). Adult animals were food deprived to 85-90% of their original weights. Adolescents were food deprived to a target weight of 85-90% of the weight of their same sex litter mate to compensate for growth; however, occasionally an animal would fall between 80-85% before compensatory feeding. Raw, hulled, unsalted sunflower seeds were used as a reward for all delayed alternation training and testing. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and concurred with all NIH guidelines.

### ***Apparatus***

All training and testing took place in a T-maze with 3 equal arms (10 cm x 45 cm with 15 cm high walls) made of black Plexiglas. There was a black Plexiglas start box that was 15 cm x 26 cm with walls that were 15 cm high and had a translucent lid that opened and closed in order to place the animal in the box. The start box was attached to one of the arms and a sliding vertical door opened to allow the animal to enter the maze. Sunflower seeds were purposely spread on the table around the outside of the maze to counteract any olfactory cues.

### ***Delayed Spatial Alternation Task***

Throughout all phases of training and testing, 2 sessions were completed per day because of the lengthy training period this task requires and the limited time rats are in the adolescent

period (postnatal day 28-42; Spear, 2000). Training and testing sessions occurred twice daily, once between 9-1pm and again from 4-7pm and consisted of 10 trials. All procedures utilized a continuous-trials procedure. Training consisted of exposure to the maze, shaping, 0-delay training and pretesting. Animals began all training and testing trials in the start box which was separated by a Plexiglas sliding door that was opened at the beginning of each trial to allow the animals to enter the T-maze.

Animals were first exposed to the maze 4 times across 2 days. During this time several sunflower seeds were placed in the both arms of the T-maze and the animals was allowed to explore the maze for 10 minutes. This was done to decrease the novelty of the maze and to get the animals used to eating sunflower seeds in the two arms of the T-maze. Next animals began shaping which was simply forced alternation. Animals were placed in the start box, the vertical door was raised and then the animal was forced to go either right or left depending on which arm was blocked. Each arm was baited with one sunflower seed and subsequent trials blocked the opposite arm of the previous trial. Animals were then removed after the sunflower seed was consumed and placed back in the start box where the next trial immediately began. The first blocked arm was alternated between sessions so that alternation began both on the left and the right. Shaping consisted of 6 untimed sessions of 10 trials each session across 3 days, after which training was initiated.

Training was similar to shaping except the animals were not forced to go left or right on any trial. During the first trial animals choose freely and were rewarded with one sunflower seed either way they went. The free choice trial was used to observe any side-bias during training and testing. In the subsequent ten trials animals were rewarded with one sunflower seed when they entered the alternate arm. Rats had to fully enter one arm in order for a choice to be

recorded and at this time animals were confined to that arm of the maze. All trials were continuous trials without delays. An animal reached criterion when it completed 13 sessions or achieved 8 out of 10 alternations correctly in 3 consecutive sessions to avoid over training. If an animal did not leave the start box in 5 minutes anytime during the session the entire session was halted and not counted in the overall analysis of training. All animals that behaved in this way did eventually leave the start box regularly and performed well in subsequent trials (80% or better).

Before testing, a pretest was implemented where no or small delays (0, 5, 10, 15 seconds) were introduced for 2 sessions of 10 trials each. This was done because the experimenter observed during a pilot study that animals would perform poorly on the first few sessions of testing because of the introduction of the delays. After pretest sessions were completed animals began testing.

Testing was similar to training except all delays were implemented (5, 10, 15, 30, 60, and 90 seconds). The average age for the beginning of testing was: adult females =  $90 \pm 1.5$  days, adult males =  $91 \pm 1$  days, adolescent females =  $34 \pm 2$  days, adolescent males =  $35 \pm 1$  days. Animals completed 15 sessions containing 10 trials per session. Delays were presented once or twice during each session but the same delay was never run consecutively. Within each session a different order of delays was presented that was standard across subjects. Testing also began with one free choice trial similar to training in which there was no delay. Delays began when the animal was placed in the start box after the animal consumed all of the reward from the free choice trial and continued throughout the remaining 10 continuous trials. The first 23 trials of each delay that were completed by each animal were included in the analysis. During testing, if the animal did not exit the start box within 10 seconds of the delay the animal was still allowed

to choose an arm but the trial was not included in the analysis. This occurred in 8 animals (2 adults and 6 adolescents). However their overall performance did not significantly differ between other animals within their group.

The number of errors was recorded in training and testing as well as whether or not that error was perseverative. A perseverative error was defined as the selection of an arm that was not rewarded in the last choice. For example, animals would make one error and then continue choosing that same arm. Since the present design used continuous-trials this equals two errors in a row (i.e. left, right, left, left, left = 2 errors and 1 perseverative error).

### ***Statistical Analysis***

The number of sessions to reach criterion in training was compared between the groups using a 2-way ANOVA (sex x age) with litter as a covariate. Post hoc 1-way ANOVAs were performed between the groups in order to compare interaction effects. Total errors in testing were analyzed by performing a repeated measures 3-way ANOVA (sex x age x delay) using litter as a covariate. Preplanned post hoc univariate F tests for age were used to compare the differences at the various delays. Additionally a repeated measure 3-way ANOVA was also performed (sex x age x delay) using litter as a covariate to analyze perseverative errors per trial in both training and testing. All statistical analyses were performed using Systat 12.0.

## **Results**

### ***Side-bias***

Analysis of the free choice in training and testing revealed that one particular side was not chosen over the other by animals as a whole. However within animals, there were several adult and adolescent animals that preferred one side over the other. The criterion for this measure



was if an animal chose the left or right side more than 67% of the trials. In training, 44% of female adults, 50% of male adults, 63% of female adolescents, and 0% of male adolescents chose the one side more. In testing 67% of female adults, 50% of male adults, 63% of female adolescents, and 38% of male adolescents chose one side more. Interestingly even though side-bias rates were high in both training and testing it occurred, only 2 adult females, 1 adult male, and 3 adolescent females had a side-bias during both testing and training.

### ***Training***

Figure 2.1 illustrates the number of sessions to reach criterion in the training phase for each group. A two-way ANOVA analysis revealed an age x sex interaction [ $F(1,28) = 5.057$ ;  $p = 0.033$ ; see Figure 2.1]. Post hoc analysis of age found a significant effect only in females ( $p = 0.013$ ) with adult females reaching criterion in fewer trials than adolescent females. A post hoc test also showed a sex difference in adolescent animals where adolescent males completed training faster than did the females ( $p = 0.031$ ). There was also a significant overall effect of the covariate, litter [ $F(1,28) = 8.751$ ;  $p = 0.006$ ]. A linear regression was also performed on the percentage correct from trial to trial; however this showed no significant differences between the groups. This may be due to the fact that most animals performed well in training at the beginning of the task. A further analysis was done to investigate differences in spontaneous alternation rates by observing any differences at the onset of training (session 1 and session 2; data not shown). This analysis reported an overall age effect [ $F(1,28) = 5.469$ ;  $p = 0.027$ ] and sex effect [ $F(1,28) = 4.580$ ;  $p = .041$ ] as well as a litter effect [ $F(1,28) = 6.837$ ;  $p = 0.0014$ ]. These results revealed that females as well as adolescence performed worse at the onset of training. The differences appear to be mainly driven by the adolescent females only achieving an

average of 56% correct in the first 2 sessions whereas the rest of the groups performed well above chance (female adults = 69%; male adolescents = 70%; male adults = 69%).

Perseverative errors were measured in the training sessions by analyzing the number of perseverative errors per total number of trials completed (Figure 2.3) because not all animals completed the same amount of trials. A two-way ANOVA analysis found a significant age effect [ $F(1,28) = 8.551$ ;  $p = 0.007$ ] that indicated adolescents perseverated more than adults. A significant overall sex difference was also found [ $F(1,28) = 8.243$ ;  $p = 0.008$ ] demonstrating that females perseverated more than males.

### ***Testing***

When delays were added during the testing phase, an overall delay effect indicated that less correct choices occurred with longer delays [ $F(4,150) = 11.507$ ;  $p < 0.001$ ; see Figure 2.2a]. Additionally there was a significant age effect [ $F(1, 28) = 4.306$ ;  $p = 0.047$ ] (adults > adolescents) and a strong trend for a time x age interaction [ $F(5, 140) = 2.263$ ;  $p = 0.051$ ]. Post hoc comparisons revealed significant differences between adolescents and adults on the 15 second delay ( $p < 0.01$ ) and the 60 second delay ( $p < 0.05$ ). No significant sex differences were found however it appears that the age difference was stronger in males than females (Figure 2.2b).

There was an overall age effect in perseverative errors indicating the average number of perseverative errors per trial was significantly higher in adolescents than adults [ $F(1,28) = 9.339$ ;  $p = 0.028$ ; Figure 2.3]. In fact adolescents committed 44% more perseverative errors than adults overall. There were no sex differences in perseverative errors at either age during delay testing.

## Discussion

Several studies have reported that the structure of the rat mPFC is maturing throughout adolescence, however there have been very few studies investigating the behavioral functioning of the adolescent mPFC. The current study compared the performance of adolescents and adults on the PFC-dependent task, the delayed alternation task. The results demonstrated that adolescent rats can perform this task relatively well, but they commit significantly more errors during 15 and 60 second delays and more perseverative errors overall than adults. Even though both groups performed worse on the task as delays increased, adolescents decline at a shorter delay than adults. To the authors' knowledge this is the first study to directly compare adolescents and adult rats of both sexes in a delayed alternation task. Overall these results are consistent with studies in humans and primates showing that PFC-dependent behaviors have not reached adult levels during adolescence (Alexander and Goldman, 1978; Anderson et al, 2001; Casey et al, 1997; Levin et al, 1991). In rats, studies that have compared adolescent and adults in cognitive tasks have shown similar results (Niemi and Thompson, 1980; Schenk, 1985) but others have not (Bauer, 1980; Chambers et al, 1996).

There are several dissociations between the age and sex groups in performance on the delayed alternation task and on the other behavioral measures that make simple solitary explanations difficult. Adolescents performed more poorly at only some delays so it is unlikely that a general function such as increased activity or exploration is the sole explanation (Spear and Brake, 1983). It should also be noted that adult females are more active than adult male rats, but there are no significant sex differences on delay testing. While adolescents committed more perseverative errors in delayed testing when compared to adults, they also had more perseverative errors in training, where male and female adolescents differed on the number of

trials to criterion. A side-bias is also dissociated from the performance measures in both training and testing in that there was a very low incidence of side-bias in adolescent males in comparison to the other groups.

These deficits in the performance of the delayed alternation task are likely related to the immaturity of the mPFC. The ongoing development of the rat mPFC has been reported by several anatomical studies in which there is pruning of neurons as well as continued changes in connectivity (Cressman et al, 2010; Cunningham et al, 2002; Markham et al, 2007), and lesions to the PFC increase perseverative errors in studies similar to the present study (Dias and Aggleton, 2000; Ragozzino et al, 2003). In addition, neurotransmitter alterations occur throughout adolescence and are known to impact delayed response tasks. For instance, there are changes in NMDA receptors (Jablonski et al, 2010; Watson et al, 2009), the cholinergic system (Castro et al, 1990) and the dopaminergic system (Mizoguchi et al, 2009; Verma and Moghaddam, 1996; Vijayraghavan et al, 2007).

No significant sex differences were detected in the performance of the delayed task in the number correct or perseverative errors committed. Thus performance did not reflect the sex differences in neuron number in the adult mPFC that has been reported by our laboratory (Markham et al, 2007). However, the lack of sex differences did correspond to other reports using the delayed spatial alternation task in adults (Corwin et al, 1981; Corwin et al, 1982). In contrast in training with no delay, female adolescents were both worse at the task and committed more perseverative errors than male adolescents or female adults. This may in part be due to their lower rate of spontaneous alternation at the beginning of training. This sex and age difference may reflect more immature structures or systems (the mPFC, the striatum, or the hippocampus), which are needed to perform this task, in adolescent females. Furthermore

because the training portion of this task was completed before puberty and testing was performed after puberty, hormones may be impacting performance. It would be interesting to investigate spontaneous alternation rates in female and male animals prior to and post puberty.

Although the version of the delayed alternation task used in this study is not novel (Markowska and Savonenko, 2002), it is possible that other versions of the delayed alternation task may be more sensitive for detecting differences between adults and adolescents. In a discrete-trials version of the task (not continuous), Mizoguchi and colleagues (2009) found differences between adults and aged rats using similar delays, but the delays were presented in a cluster of trials instead of being randomized within a session as in the present study. In this paradigm, the adult animals performed better than animals in the present study especially at 60 seconds (~77% correct compared to 64% in present study). It is possible that our testing regimen is slightly more difficult because of the unpredictability of the delay due to the randomization of trials and/or from the lack of the information trial, which may serve to reset to the rat's working memory. On the other hand, Green and Stanton (1989) compared these two versions of the delayed alternation task (discrete versus continuous trials) and found that the ontogeny of these tasks differed such that male rats could perform continuous trials earlier than discrete trials (Green and Stanton, 1989).

The present study found that adolescents performed the delayed alternation task with an increased number of errors at certain time points and committed significantly more perseverative errors than adults. These deficits could be related to the immaturity of the mPFC or other related brain areas, such as the hippocampus or the BLN. Although many studies have documented that the brain is still immature in the adolescent period, there have been few studies directly relating adolescent development to behavior. More work is needed on the functional consequences of

continuing brain development in adolescence in order to further explore mechanisms for psychopathologies such as depression, schizophrenia, and drug and alcohol addictions that appear during this critical time.

## Figures

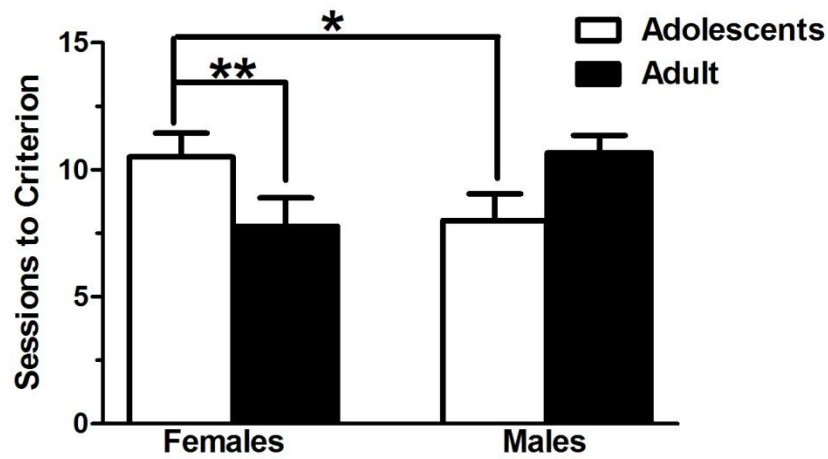


Figure 2.1 Acquisition of the task without delays. ANOVA analysis revealed a sex x age interaction with female adolescents performing significantly worse than male adolescents and female adults. \* =  $p < 0.05$ ; \*\*  $p < 0.01$

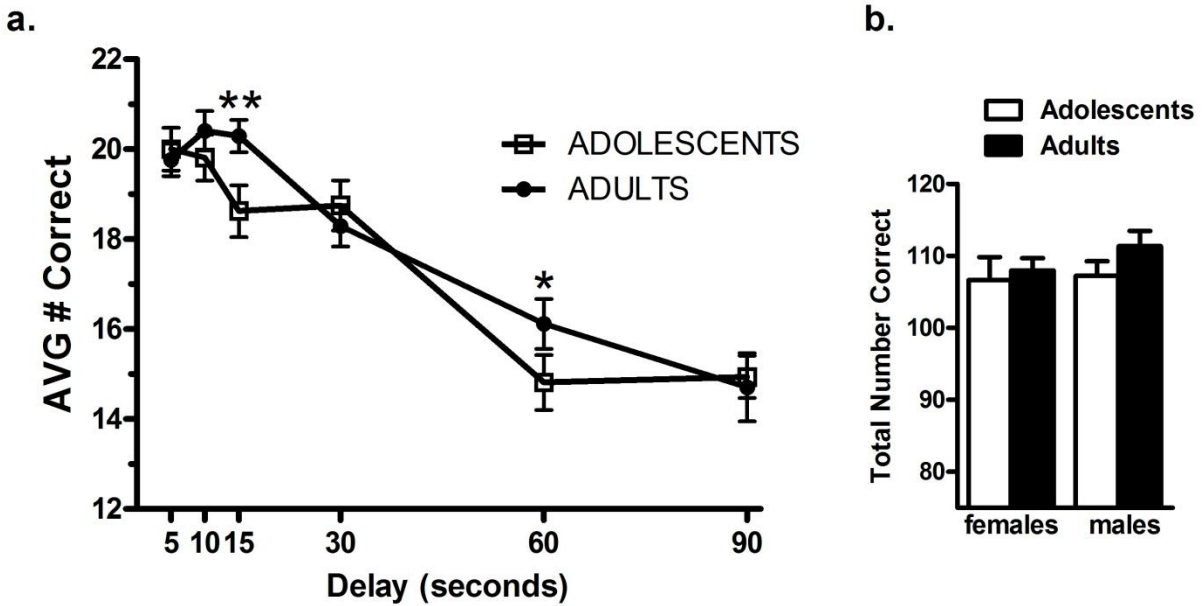


Figure 2.2 Average number of correct choices during delay testing a.) across delays and b.) total correct for females and males. Adolescent rats had a deficit in performance during 15 and 60 second delays when compared to adults. There were no significant sex differences. \*\* $p < 0.01$ :  
\*  $p < 0.05$



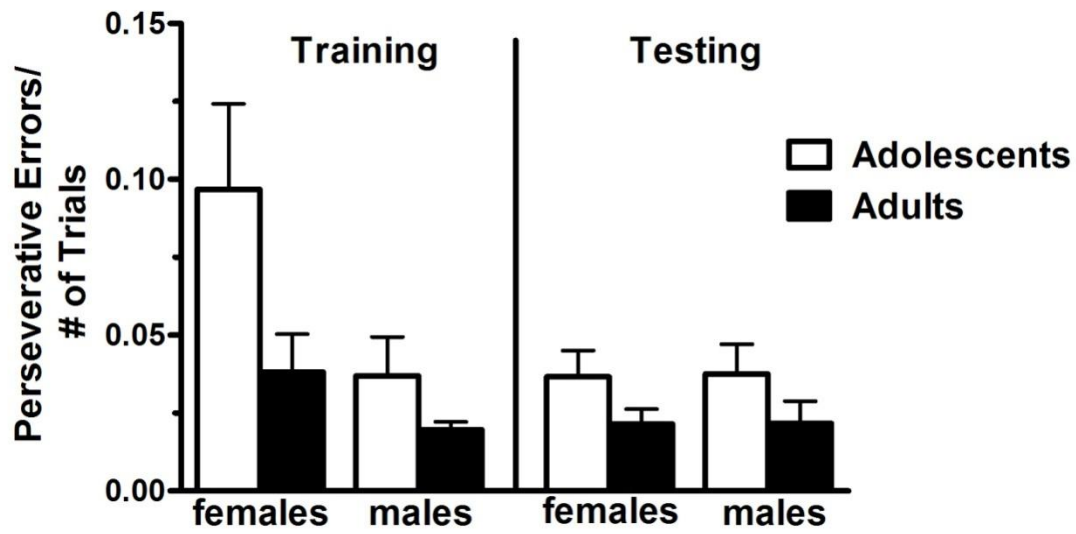


Figure 2.3 Average number of perseverative errors per trial in training and testing for females and males. There was a significant age effect in both training ( $p < 0.05$ ) in testing ( $p < 0.01$ ) and a sex difference ( $p < 0.01$ ) for training only.

## **Chapter 3**

# **Dendritic Remodeling in the Rat Adolescent Medial Prefrontal Cortex and the Basolateral Amygdala**

## Introduction

The amygdala and prefrontal cortex (PFC) are neural regions that are important for cognition and emotional regulation (Pare, 2003; LeDoux, 2003; Uylings et al, 2003; Dias and Aggleton, 2003; Dunnett et al, 1999). Adolescents have shown deficits in behaviors associated with these areas when compared to adults. In human adolescents, working memory, attention, and processing speeds are not yet at adult levels (Graber et al, 1996; Keating, 1990, Levin et al, 1991; Anderson et al, 2001) and similarly, rats have deficits in working memory, spatial memory and other memory tasks (Koss et al, 2011; Schenk, 1985; Niemi & Thompson, 1980). In studies involving emotional stimuli, human adolescents have greater amygdala activation to fearful faces as assessed by fMRI (Monk et al, 2003) but decreased activation in the mPFC (Killgore et al, 2001) when compared to adults. In rodents, performance in amygdala-dependent tasks, such as fear conditioning and behavioral tests of anxiety, improve throughout the adolescent period (Hefner and Holmes, 2007; Wiedenmayer, 2009; Ernst and Fudge, 2010). Moreover, our laboratory has shown that adolescent rats have deficits in an amygdala-dependent appetitive task (Rubinow et al, 2009a). These studies all suggest that functionally the mPFC and the amygdala are not fully developed functionally until adulthood.

Neuroanatomically, human MRI studies have reported structural changes in both of these neural regions throughout the adolescent period. In the PFC, gray matter volume increases throughout childhood with a peak around 11-12 years old, followed by a decrease throughout adolescence until approximately age 22 (Giedd et al, 1996b; Jernigan et al, 1991; Sowell et al, 1999). In the amygdala, gray matter volume increases throughout childhood and adolescence (Merke et al, 2003; Giedd 1996a; Schumann et al, 2004). Underlying mechanisms of these volume changes could be axon and dendrite remodeling, as well as changes in neuron number. In

rats, our laboratory has described significant decreases in the number of neurons from adolescence (postnatal (P) P35) to adulthood(P90) in the ventral portion of the medial prefrontal cortex (mPFC) and the basolateral amygdala (BLN)(Markham et al, 2007; Rubinow and Juraska, 2009).Axon refinements have also been reported to occur within the connections between the two structures. BLN efferents to the mPFC are sproutingbetween the adolescent period and early adulthood (P35-P65), creating more axon terminals (Cunningham et al, 2002),and PFC efferents to the BLNare pruningbetween P45 and P90 (Cressman et al, 2010).

In the mPFC, dendritic arborization and spines have only been assessed during adolescence in layer 3 pyramidal where an increase in dendritic arborization with some decreases in spine density was found (Markham et al, 2012). Paralleling the decreases in spines, synapsesduring adolescence have been reported in the mPFC of both humans (Huttenlocher, 1979) and non-human primates (Bourgeois et al, 1994, Anderson et al, 1995). In comparison, the BLN has been less studied than the mPFC. To date, dendritic arborization and dendritic spine density across adolescence have not been examined in the BLN. In our laboratory, we observed a decrease in neurons, as well as glia,in the adolescent BLN which was not associated with losses of volume (Rubinow and Juraska, 2009). One explanation for this finding is that dendritic processes may be growing, thus replacing the volume that previously contained neurons and glia. The BLN is sometimes classified as cortical-likebecause of its embryonic origins and connections (McDonald, 1998). Others have examined more striatum-like nuclei in the rodent amygdala. For instance, in the medial amygdala, which is more involved in sex behavior, the volume and the number of neurons increases during the adolescent periodin male Syrian hamsters (Romeo and Sisk, 2001; Ahmed et al, 2008). Additionally, dendrites and

dendritic spines have been shown to prune in the medial amygdala, similar to the mPFC, in this structure throughout adolescence (Zehr et al, 2006).

In the subset of studies examining both sexes differences have been found in the mPFC and the amygdala. In human MRI studies the volume of the PFC peaks earlier in females than in males (Giedd et al, 1999). In rats, the mPFC has a significant reduction of neurons between adolescence and adulthood, which is greater in females and is layer specific (Markham et al, 2007). This loss results in a sex difference in the number of neurons in the adult mPFC that was not present during adolescence (Markham et al, 2007). Sex differences were not detected in adolescent neuron loss or in the number of neurons in the rat BLN (Rubinow and Juraska, 2009), but imaging data in humans has found that the volume of the whole amygdala increases more in males than females across adolescence (Merke et al, 2003; Giedd 1996a; Schumann et al, 2004). Moreover there is evidence in the rat for sex differences in the pruning of dendritic spines occurring in the visual cortex throughout early development (Munoz-Cueto and Ruiz-Marcos, 1994). Behaviorally, our laboratory has not detected any sex differences in mPFC- or BLN-dependent tasks among adolescents or adults (Koss et al, 2011; Rubinow et al, 2009a). However, others have shown that adult females have reduced extinction in fear conditioning (Baran et al, 2009) as well as in a Pavlovian approach task where adult females showed the same deficits, but female adolescents demonstrated incomplete extinction when compared to male adolescents (Hammerslag et al, in press). Male adolescent rats have larger corticosterone release accompanied by a shorter recovery time after repeated restraint stress than male adult rats (Romeo et al, 2006; Doremus-Fitzwater et al, 2009) which does not occur in females (Doremus-Fitzwater et al, 2009). Together, this may indicate functional sex differences in the basolateral

amygdala and/or mPFC appearing after or during adolescence, and also emphasizes the importance of observing both sexes within adolescent studies.

The current study examines the changes in dendritic arborization and dendritic spines in the mPFC and the BLN prior to and during adolescence in both sexes at 3 ages: juveniles (P20), adolescents (P35) and in adults (P90). We hypothesize that in the mPFC dendritic arborization and spine density will increase between the juvenile and adolescent ages, mimicking the volume changes previously found in rats as well as humans (Markham et al, 2007; Giedd et al, 1996b). Between adolescence and adulthood, we predict a pruning of dendritic spines and dendrites. Sex differences may occur similar to that seen in neuronal loss where females lose more neurons than males (Markham et al, 2007). In the BLN, we expect the dendrites and dendritic spines to increase across the juvenile and adolescent period. This is due to our laboratory's previous data indicating increases in volume in the juvenile phase with no neuron number changes and the losses of neurons in the adolescent period accompanied by no volume changes (Rubinow and Juraska, 2009).

## **Methods**

### ***Subjects***

All rats used were first generation descendants from Long Evans breeders in our vivarium originally obtained from Simonsen Laboratories (Gilroy, CA). Animals in the youngest group were sacrificed at P20. Adolescent and adult groups were weaned at P24 and then housed 2-3 per cage, handled weekly, kept on a 12-h light-dark cycle (lights off at 20:00h) and given food and water *ad libitum* until they were sacrificed at P35 and P90. Each group contained 5-8 animals per group. Animal care and experimental procedures were in accordance

with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Illinois Urbana-Champaign.

### ***Histology***

All rats were deeply anesthetized with a lethal dose of sodium pentobarbital (100mg/kg; Sigma, St. Louis, MO). Golgi impregnation methods were performed similar to that described in previous work from our laboratory (Markham and Juraska, 2002; Rubinow et al, 2009b) as well as others (Glaser and Van der Loos, 1981). Brains were harvested; olfactory bulbs and cerebellum removed, and then brains were immersed in 100ml of Golgi-Cox solution for 19-20 days. Golgi-Cox solution was prepared from 5% solutions of potassium dichromate, mercuric chloride, and potassium chromate, in a 5:5:4 volume part ratio. Test-slices were cut at the level of the basolateral amygdala to verify that neurons were well-filled. The two poles, anterior and posterior, were separated by cutting the brain at approximately 5mm from the anterior pole. Both halves were dehydrated, embedded in a 12% celloidon solution, hardened and stored in butanol until sectioning. Brains were coded to ensure that the experimenter was blind to the animal's group identity. Embedded brains were sliced on a microtome at a thickness of 150um in order to capture the entire extent of the dendritic tree of neurons. After slicing, tissue slices were developed by immersions in the following solutions: ammonium hydroxide, photographic fixative, increasing ethanol solutions for dehydration, and cleared in xylene. Sections were then mounted on slides with permount and coverslipped.

### ***Identification of BLN neurons***

The basolateral nucleus location was identified by morphological characteristics as previously described in Rubinow et al (2009b). Additionally, Nissl stained tissue from animals of corresponding ages were examined to assess boundaries (Rubinow and Juraska, 2009).

The tear-dropped shape of the basolateral complex (BLA) was distinguished in Golgi-stained tissue by the presence of white matter both medial and lateral to the structure. In anterior sections, the lateral amygdala cells were distinguished from BLN neurons by size and location. In the middle to posterior sections of the BLN, these characteristics could not be determined with confidence; therefore, the majority of cells chosen for this study were from the anterior half of the BLN.

Principal or class I pyramidal cells were sampled from the BLN which have been described by McDonald (1982). These neurons represent ~85% of neurons in the BLN (Muller et al, 2006) and are readily impregnated by the Golgi-Cox stain. They can be distinguished from spine-sparse interneurons or stellate-like shaped class II neurons which may also be impregnated by Golgi-Cox stain. The class I pyramidal neurons have irregular orientations and large dendritic trees that exhibit numerous spines and usually pyramidal or semi-pyramidal cell bodies (Figure 3.1b). They contain 5-7 primary, or first order, dendrites where 1-2 of these dendrites display a thicker phenotype than the other primary dendrites. Therefore, due to individual differences within neurons all dendrites were analyzed together, unlike mPFC neurons that have clear basilar and apical dendritic regions.

#### ***Identification of layer 5 mPFC neurons***

The anterior boundary of the mPFC was identified by the first section containing cortical white matter, whereas the posterior boundary was identified as the first section containing the genu of the corpus callosum. The ventral boundaries within the mPFC containing the prelimbic (PL) and infralimbic prefrontal cortex (IL) were defined by prior work from our laboratory (Markham et al, 2007; Yates et al, 2008a) and by examination of Nissl stained tissue from a separate group of animals. For sampling, cell bodies had to be clearly in layer 5 which is widens



(~200um) in the PL and narrows through the IL (~100um). The majority of the layer 5 pyramidal neurons sampled were contained in the PL division of the mPFC, and all cell bodies were 500-700um from the midline. These criteria were determined by measurements taken from the sample of prior Nissl stained tissue containing all 3 ages assessed in this study (P20, P35 and P90). Additionally, cellular characteristics such as cell body size and branching patterns also distinguished the cell bodies of layer 5 neurons. Layer 5 pyramidal neurons in the cortex have pyramidal cell bodies with one thick apical dendrite that is oriented toward the pial surface of the cortex and either ends or forms a tuft (multiple branches) in layer 1 of the mPFC. Oblique dendrites sprout from the apical dendrite before the layer 1 tuft forms and may branch once or twice before terminating (Figure 3.4a). Basilar dendrites have thinner primary dendrites, radiate from the cell body and branch multiple times before terminating (Figure 3.3b).

### ***Quantification of the Dendritic Arbor***

Analysis of dendritic material was performed using the NeuroLucida 9.0 program (MicroBrightfield). Ten to twelve well-filled neurons in the BLN and 7-10 neurons in the mPFC (apical and basilar trees separately) were drawn in 3-dimensions using a 63x objective on a Zeiss Axioimager A1 light microscope. Neurons were only selected if they fell within the boundary parameters outlined above. All efforts were taken to ensure neurons were fully contained within a section, and minimally overlapped other stained elements. Total dendritic length (the sum of all branches), the length of terminal and bifurcating ends and the number of branches overall and by centrifugal order were assessed to investigate dendritic changes. A Sholl analysis (Sholl, 1956) was also performed. Sholl analysis counted the number of intersections made by dendritic material every 20um away from the cell body using concentric spheres, thus analyzing discrete changes in particular portions of the dendritic tree (Figure 3.1b, 3.3b, 3.4a). Measures were taken

from the entire extent of each neuron for total dendritic, terminal length and branch length in BLN neurons and basilar mPFC dendrites. Branch by centrifugal order and sholl analysis was only measured through 8<sup>th</sup> order branches and 360um away from the cell body for the BLN and 7<sup>th</sup> order and 300um away from the cell body for the basilar tree of the mPFC due to lack of dendritic material occurring past those areas of the dendritic tree. In apical dendrites of the mPFC only Sholl analysis was performed from 0-300um away from the cell body in order to limit the analysis to the oblique branches and avoid the branching inconsistencies of the layer 1 tufts of mPFC neurons. Some neurons possess a layer 1 tuft that branches abundantly but do not branch at all. All individual neuron measurements were averaged within subject then across group and all analyses were performed using the NeuroLucida Explorer 4.7 (MicroBrightfield, Inc.).

### ***Quantification of Dendritic Spines***

Spine density was quantified with a 100x objective on an Olympus BH2 light microscope with an attached camera lucida. Ten to twelve well filled segments were chosen from the BLN neurons (Figure 3.2a) and from each of the basilar and apical regions of mPFC neurons (Figure 3.2c). Only dendritic segments that were located in one plane of focus were included to avoid error in segment lengths. Each segment was approximately  $1 \pm 0.2\mu\text{m}$  in thickness and at least 10um in length. No more than 3 segments were sampled from 1 neuron and all segments were terminal segments. In the BLN, all segments were at least 40-100um away from the cell body and terminated within 50um as previously described in Rubinow et al (2009b). In the mPFC apical tree, oblique dendrites of 2<sup>nd</sup> or 3<sup>rd</sup> order segments were examined whereas in the basilar tree 2<sup>nd</sup> to 5<sup>th</sup> order terminating segments were chosen, similar to Markham and Juraska (2002). The hormonal cycle of adult females was not controlled because prior data in our laboratory

found no differences in spine density across the cycle in the BLN (Rubinow et al, 2009b) or the mPFC (Markham and Juraska, 2007). All spine density measures were calculated as an average number of spines per 10um of dendritic length per subject.

### ***Statistical Analyses***

All analyses were performed using Systat 12.0 or Graph Pad Prism 5.0. Two-way ANOVAs (sex, age) were used for all analyses except for Sholl and order analyses, where three-way repeated measure ANOVAs (sex, age, sphere or order) were performed, with sphere and order as the repeated measures. In all ANOVAs, litter was run as a covariate. If ANOVAs were significant post-hoc t-tests were performed for further analysis of two-way ANOVAs and post-hoc Tukey tests were run for three-way repeated measure ANOVAs.

## **Results**

### ***Basolateral Amygdala***

#### ***Dendritic Arborization***

In the BLN, total dendritic length measurements revealed a significant effect of age ( $F(2,33) = 9.41$ ,  $p = 0.001$ ; Figure 3.1a) with post hoc t-tests indicating a significant increase of dendritic length from P20-35 ( $p = 0.01$ ) and a weak trend of a further increase from P35 and 90 ( $p = 0.1$ ). The lengths of terminal and bifurcating dendrites were also analyzed. This resulted in a significant age effect of terminal endings ( $F(2,33) = 13.606$ ,  $p < 0.001$ ) but not bifurcating branches (Table 3.1). Significant increases were found in terminal endings between P20 and P35 animals ( $p < 0.001$ ) but not between P35 and P90. Branching analysis by centrifugal order found a significant effect of age ( $F(2,33) = 23.11$ ,  $p = 0.03$ ), order ( $F(7,231) = 162.48$ ,  $p < 0.001$ ) and a significant order by age interaction ( $F(14,231) = 1.95$ ,  $p = 0.02$ ). Interestingly, an overall

increase in the number of branches was shown between P35 and P90 but not between earlier ages, and post hoc t-tests revealed significant increases specifically at 2<sup>nd</sup> and 4<sup>th</sup> order branches ( $p < 0.05$ ; Table 3.2). There were no significant sex differences in any of these measures.

Sholl analysis in BLN neurons indicated significant effects of age ( $F(2,33) = 9.41$ ,  $p = 0.001$ ), sphere ( $F(17,561) = 218.118$ ,  $p < 0.001$ ), and an interaction between sphere and age ( $F(17,561) = 7.778$ ,  $p < 0.001$ ) (Figure 3.1c), but no sex differences. Significant age effects were found between 100um and 180um away from the cell body between P20 and P35 ( $p \leq 0.001$ ), indicating large amounts of dendritic material being added to the tree within these specific areas (Figure 3.1c). Between P35 and P90, a significant increase in dendritic material was found only at the first concentric sphere (20um;  $p = 0.04$ ).

#### *Dendritic Spines*

A statistical effect of age was found when analyzing dendritic spine density in BLN terminal dendrites ( $F(2,33) = 3.748$ ,  $p = 0.03$ ; Figure 3.2). Post t-tests revealed that spine density increased from P20 and 35 ( $p = 0.004$ ) with no differences between P35 and 90. Similar to dendritic arborization measures, there were no significant effects of sex on dendritic spine measures.

#### *Medial Prefrontal Cortex*

##### *Basilar Dendrites*

In the basilar region of the mPFC, there was a significant age effect in total dendritic length ( $F(2,30) = 24.99$ ,  $p < 0.001$ ; Figure 3.3a), with post hoc t-tests indicating a significant increase in total dendritic length between P20 and P35 ( $p < 0.001$ ), but no significant effects between P35 and P90. Analyses of terminal and bifurcating branches found significant effects of age ( $F(2,30) = 24.16$ ,  $p < 0.001$ ;  $F(2,30) = 6.92$ ,  $p < 0.001$  respectively; Table 3.1). T-tests

revealed that terminal ( $p < 0.001$ ) and bifurcating ( $p < 0.001$ ) dendrites were significantly longer at P35 when compared to P20, and there was a trend for smaller terminal lengths at P90 when compared to P35 ( $p = 0.08$ ). The number of branches was also analyzed and there were no significant differences of age, but there was an overall effect of order ( $F(7,210) = 251.51$ ,  $p < 0.001$ , Table 3.2). No significant sex differences were found in any of these measures.

Sholl analysis of basilar mPFC neurons resulted in a significant overall effect of age ( $F(2,30) = 28.10$ ,  $p < 0.001$ ), sphere ( $F(14,420) = 298.22$ ,  $p < 0.001$ ), and a sphere by sex by age interaction ( $F(28,420) = 1.95$ ,  $p = 0.003$ ; Figure 3.3c and 3.3d). T-tests indicated growth between P20 and P35 throughout most of the tree in both sexes (60um to 200um;  $p < 0.05$ ). In contrast, between the later ages of P35 and P90, pruning was apparent 60um to 100um away from the cell body only in females ( $p < 0.05$ ).

#### *Apical Oblique Dendrites*

As depicted in Figure 3.4, a Sholl analysis resulted in an overall effects of age ( $F(2,30) = 24.85$ ,  $p < 0.001$ ), sphere ( $F(14,420) = 19.63$ ,  $p < 0.001$ ), and a significant sphere by age interaction ( $F(28,420) = 3.725$ ,  $p < 0.001$ ). However, no sex differences were observed. T-tests revealed that there was significant growth between P20 and P35 at 60um to 240um away from the cell body ( $p < 0.01$ ), very similar to the basilar tree of the mPFC and the BLN. There were no age differences between P35 and P90.

#### *Dendritic Spines*

Analysis of the density of basilar dendritic spines resulted in a significant age effect ( $F(2,27) = 5.546$ ,  $p = 0.01$ ; Figure 3.5a). Similar to the dendritic arbor, there were increases of spines between P20 and P35 ( $p < 0.01$ ) in both sexes. Additionally, there was a significant

decrease in spines after P35 ( $p = 0.03$ ). These results indicate a pruning of spines in the basilar region of layer 5 neurons.

In the apical oblique branches, an age effect was found ( $F(2,27) = 11.248$ ,  $p = 0.007$ ; Figure 3.5c) resulting in an increase of spines that occurred in both sexes similar to the basilar region between P20 and P35 ( $p < 0.01$ ). Comparable to the basilar tree, there was a slight decrease in spines between P35 and P90 but this decrease did not reach significance ( $p = 0.08$ ). No sex differences were found in apical spine density.

## **Discussion**

This is the first study to report changes in the dendritic arbor and spines of pyramidal neurons in the BLN and in layer 5 of the mPFC during the juvenile (P20 and P35) and adolescence (P35 and P90) periods in both sexes. In juveniles, significant growth was found in dendritic material and dendritic spines in both structures. These findings are consistent with our hypothesis as well as prior research performed in layer 3 pyramidal neurons of the mPFC and visual cortex (Juraska, 1982; Markham et al, 2012). After the juvenile period, the dendritic changes in the two structures diverged. In the mPFC, pruning occurred during adolescence in the basilar spines of both sexes. These results in the adolescent mPFC are consistent with our predictions as well as a previous study that reported a dendritic spine loss in layer 3 pyramidal neurons in females (Markham et al, 2012). Dendritic material was also pruned in the mPFC between P35 and P90 which differed from a report showing an increase in dendritic material in layer 3 of the mPFC between P30 to adulthood (Markham et al, 2012), suggesting a laminar difference in the adolescent development within the mPFC. Neurochemically, both pyramidal neurons found in layers 3 and 5 have similar levels of innervations from monoamine

and cholinergic axons, but there have been reports of differences in receptor levels as well as receptor subunits (Heidbreder and Groenewegen, 2003; Poorthuis et al, 2012) and different responses to neurotrophins during early development (McAllister et al, 1995). By comparison, the dendritic material in layer 3 and 5 pyramidal neurons of the visual cortex stays stable after P30 and dendritic spine density only decreases within layer 3 (Juraska, 1982). In contrast to the mPFC, there was no dendritic pruning in the BLN during adolescence, but there was an increase in dendritic branching that was not accompanied by an increase in the total length of the dendritic tree which indicates dendritic remodeling. Also no sex differences were seen which is similar to our previous findings in the adolescent BLN (Rubinow and Juraska, 2009). The lack of dendritic growth in the BLN did not explain previous findings demonstrating no volume changes after significant neuronal and glia loss (Rubinow and Juraska, 2009). This may suggest the involvement of other changes in the neuropil of BLN during adolescence, such as the number of axons, axon terminals and/or the amount of myelination.

Most excitatory synapses occur on dendritic spines (Harris, 1999). Therefore, the changes in spine density, dendritic lengths and branching illustrate remodeling of synapses throughout adolescent development, especially in excitatory synapses. Adolescent synaptic remodeling has also been shown in previous studies indicating decreases in overall synapses of the primate PFC (Bourgeois and Rakic et al, 1993; Bourgeois et al, 1994; Huttenlocher, 1979; Huttenlocher and Dabholkar, 1997). Additionally, axon sprouting occurs in the rat mPFC (Cunningham et al, 2002) accompanied by axon pruning in the rat BLN (Cressman et al, 2010) and losses of neurons in the rat mPFC and BLN (Markham et al, 2007; Rubinow and Juraska, 2009). Functional effects of these neuroanatomical modifications, including those described in the current study, are difficult to determine. Electrophysiologically, previous

literature have shown that adolescent rat mPFC neurons respond immaturely to some neurotransmitter signaling (Tseng and O'Donnell, 2005; 2007a; 2007b; Heng et al, 2011). These disparities have also been alluded to in human fMRI studies in which adolescents have less activation in the mPFC after the presentation of fearful faces (Killgore et al, 2001). In the BLN, one study found no differences in baseline neuronal activity in BLN pyramidal neurons when compared to adults, but after restraint stress the adolescent BLN recruited more pyramidal neurons than the adult BLN causing more activation in this structure (Zhang and Rosenkranz, 2012). This parallels the increase of activity shown in fMRI of the human amygdala after viewing fearful faces (Monk et al, 2003). Also, concurrent to the neuroanatomical and electrophysiological changes, many neurotransmitter systems change across the juvenile and adolescent period. In the mPFC, where the majority of adolescent research has been performed, NMDA receptors peak at P28 and then decline by age P60 (Insel et al, 1990), GABA<sub>A</sub> receptors decrease in sensitivity (Kellogg et al, 1993), and the GABA/B<sub>DP</sub> receptor complex increases in sensitivity (Kellogg et al, 1993; Primus and Kellogg, 1991). Both dopaminergic and cholinergic innervation increase in the mPFC throughout adolescence and dopaminergic receptors prune significantly (Gould et al, 1991; Kalsbeek et al, 1988; Andersen et al, 2000). Together, all of these neural changes during adolescence, including those demonstrated within this study, may be responsible for the changes in prefrontal cognitive abilities that have been shown in humans and rats (Koss et al, 2011; Casey et al, 2000; Spear, 2000; Anderson et al, 2001; Levin et al, 1991).

During adolescence, after puberty, there was significant pruning of dendritic spines in both sexes in the mPFC, but in the dendritic tree pruning only occurred in females, not males. It is possible that pruning was not identified in this study because the male mPFC and the BLN reach a peak and begin pruning after P35. Supporting later pruning, one study observed



significant pruning between P63 and P92 in BLN pyramidal neurons of males (Bergstrom et al, 2010). Along with the indications of late pruning of mPFC axons projecting to the BLN from P45 to P90 (Cressman et al, 2010), this suggests there may be late dendritic retraction in the BLN. In the mPFC, the timing of retraction may correspond with the rise of pubertal hormones. Males undergo puberty later than females (Koss, unpublished data; Castellano et al, 2011; Korenbrot et al, 1977) which may cause asynchronous timing of the dendritic changes in the mPFC. For instance, if dendritic remodeling is steroid dependent, females may prune earlier than males and if the peak of dendritic growth happens at the equivalent time as puberty onset we could be missing the peak in males by only observing P20, P35 and P90. Precise timing of the peak and the decline of dendritic and synaptic changes in both structures should be investigated in future studies.

The results of the current study demonstrate continued changes in the dendritic tree during both the juvenile and adolescent periods in two cognitive regions of the brain. During the juvenile period, the BLN and layer 5 mPFC pyramidal neurons increase similarly but deviate at adolescence when the BLN increases in the number of branches and mPFC dendrites prune mostly in females. Due to the discrete ages (P20, P35 and P90) used in this study, we cannot rule out later pruning in the BLN or in the male mPFC (i.e. growth until P45 and then loss until P90). The divergence is still intriguing given that both structures lose neurons during adolescence (Markham et al, 2007; Rubinow and Juraska, 2009), and the two structures are highly interconnected and together play important roles in regulating fear and emotional responses (Van Edén and Uylings, 1985; Amaral, 1986; Ernst and Fudge, 2011). In conclusion, the dendritic changes demonstrated in this study and in previous cellular changes shown in previous studies from our laboratory (Markham et al, 2007; Rubinow and Juraska, 2009) indicates these two

structures to be particularly late developing neural regions which may lead to specific vulnerabilities to environmental factors such as drugs and alcohol. Moreover, the onset of many psychiatric disorders appears during the adolescent period which may be due to the abnormal development or vulnerability of these two structures during this time.

## Tables

Table 3.1 Terminal and Bifurcating Lengths

	Age	Terminal	Bifurcating
Basolateral Amygdala	20	$57.4 \pm 1.8$	$27.4 \pm 0.6$
	35	$69.6 \pm 2.3^*$	$28.7 \pm 0.8$
	90	$68.2 \pm 1.2$	$28.8 \pm 0.4$
Basilar mPFC	20	$51.0 \pm 1.8$	$16.7 \pm 0.4$
	35	$68.4 \pm 2.2^*$	$19.8 \pm 0.7^*$
	90	$63.9 \pm 1.1^\#$	$18.4 \pm 0.6$

Average length of terminal and bifurcating dendritic branches (um)in the BLN and mPFC.Increases in terminal lengths and bifurcating lengths were apparent between P20 and P35 in the basilar tree of the mPFC whereas terminal lengths were only significant in the BLN. From P35 to P90 there was a trend for a decrease in terminal lengths in the basilar tree of the mPFC. In all measures no sex differences were found. Significant differences denoted by \* =  $p < 0.05$  or a trend # =  $p < 0.08$  compared to prior age.

Table 3.2 The Number of Branches Per Order

	Age (days)	# of branches							
		1°	2°	3°	4°	5°	6°	7°	8°
Basolateral Amygdala	20	5.9 ± 0.2	10.8 ± 0.3	14.2 ± 0.4	12.6 ± 0.6	7.6 ± 0.5	3.2 ± 0.3	1.1 ± 0.2	0.3 ± 0.1
	35	5.7 ± 0.1	10.6 ± 0.2	14.5 ± 0.2	12.8 ± 0.6	8.6 ± 0.7	4.0 ± 0.4	1.4 ± 0.2	0.4 ± 0.1
	90	6.0 ± 0.1	11.2 ± 0.2*	16.0 ± 0.2*	14.4 ± 0.4*	8.7 ± 0.4	4.3 ± 0.4	1.6 ± 0.2	0.6 ± 0.1
Basilar mPFC	20	6.6 ± 0.2	12.4 ± 0.3	14.7 ± 0.4	10.2 ± 0.5	4.2 ± 0.3	1.2 ± 0.1	0.2 ± 0.1	
	35	6.9 ± 0.2	13.0 ± 0.4	15.6 ± 0.5	11.1 ± 0.7	4.6 ± 0.4	1.1 ± 0.1	0.2 ± 0.04	
	90	6.6 ± 0.1	12.4 ± 0.2	15.1 ± 0.3	10.4 ± 0.4	4.6 ± 0.3	1.3 ± 0.2	0.3 ± 0.1	

Average number of dendritic branches in the BLN and the mPFC. In the BLN there was a significant overall effect of age in the number of branches only between P35 and P90. No significant differences appeared in the basilar tree of the mPFC. Significant differences are denoted by \* =  $p < 0.05$  when compared to the prior age.

## Figures

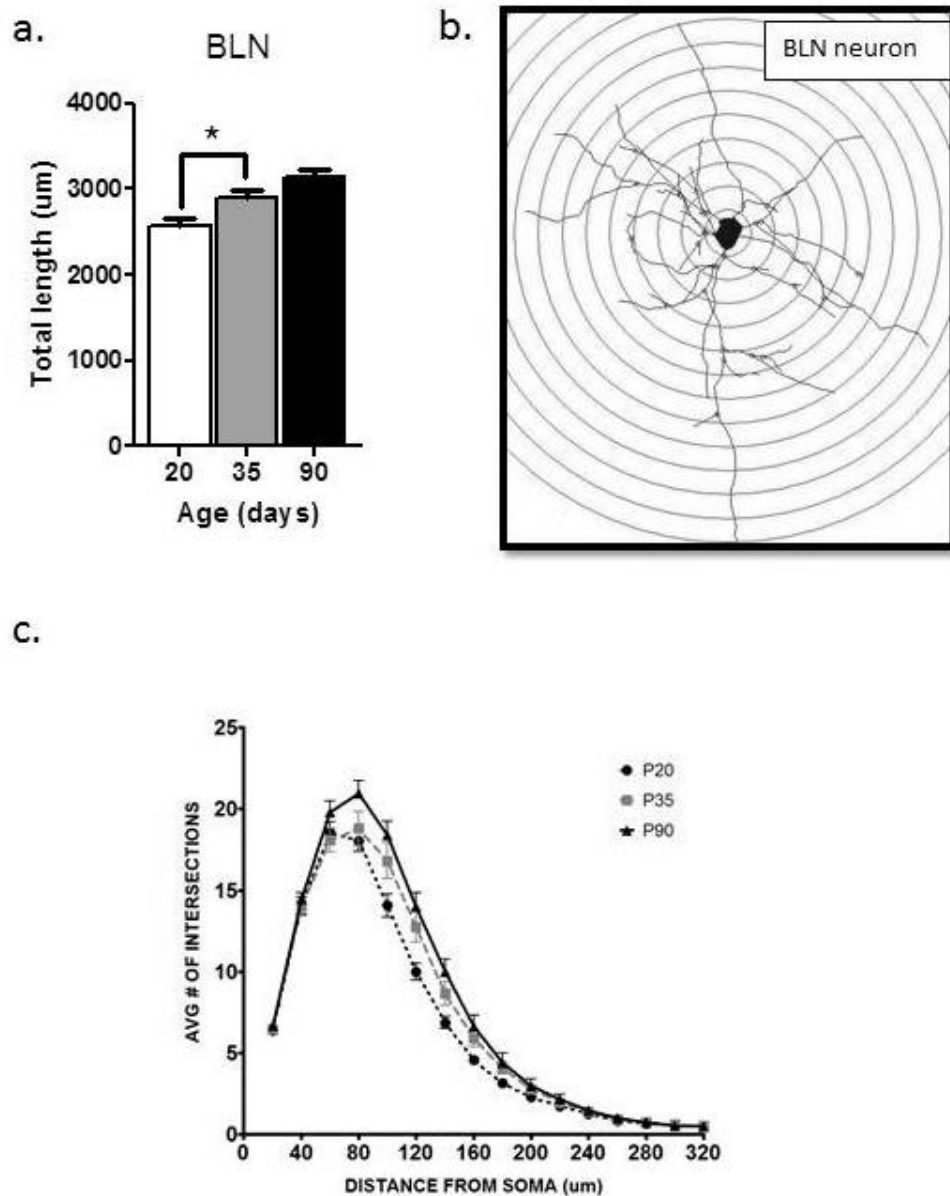
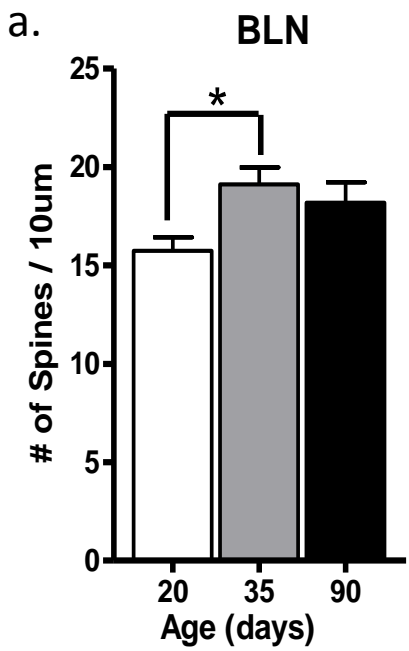


Figure 3.1 BLN dendritic tree analysis. Age comparisons of total dendritic length (a), a sample drawing of a BLN pyramidal cell and concentric Sholl spheres that are separated by 20um and radiated from the cell body (b) and Sholl results (c) are shown. These results indicate significant dendritic growth between P20 and P35; however only marginal growth from P35 and 90. \* =  $p < 0.05$



b.

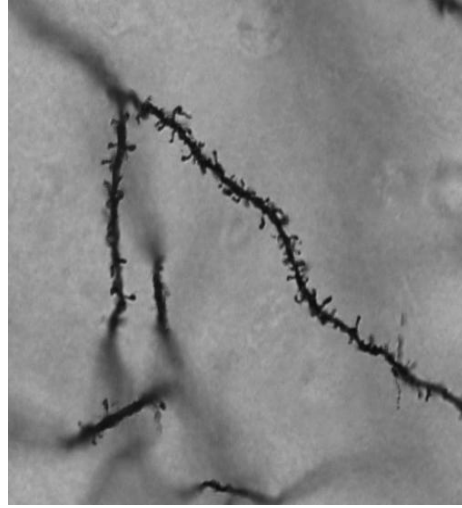


Figure 3.2 BLN measurements of dendritic spine density. Age comparisons of dendritic spines (a) and a photograph of Golgi impregnated BLN dendritic spines (b). It was found that there was an increase in spine density from P20 and P35 but no differences were found between P35 and p90, and no sex differences. \* =  $p < 0.05$

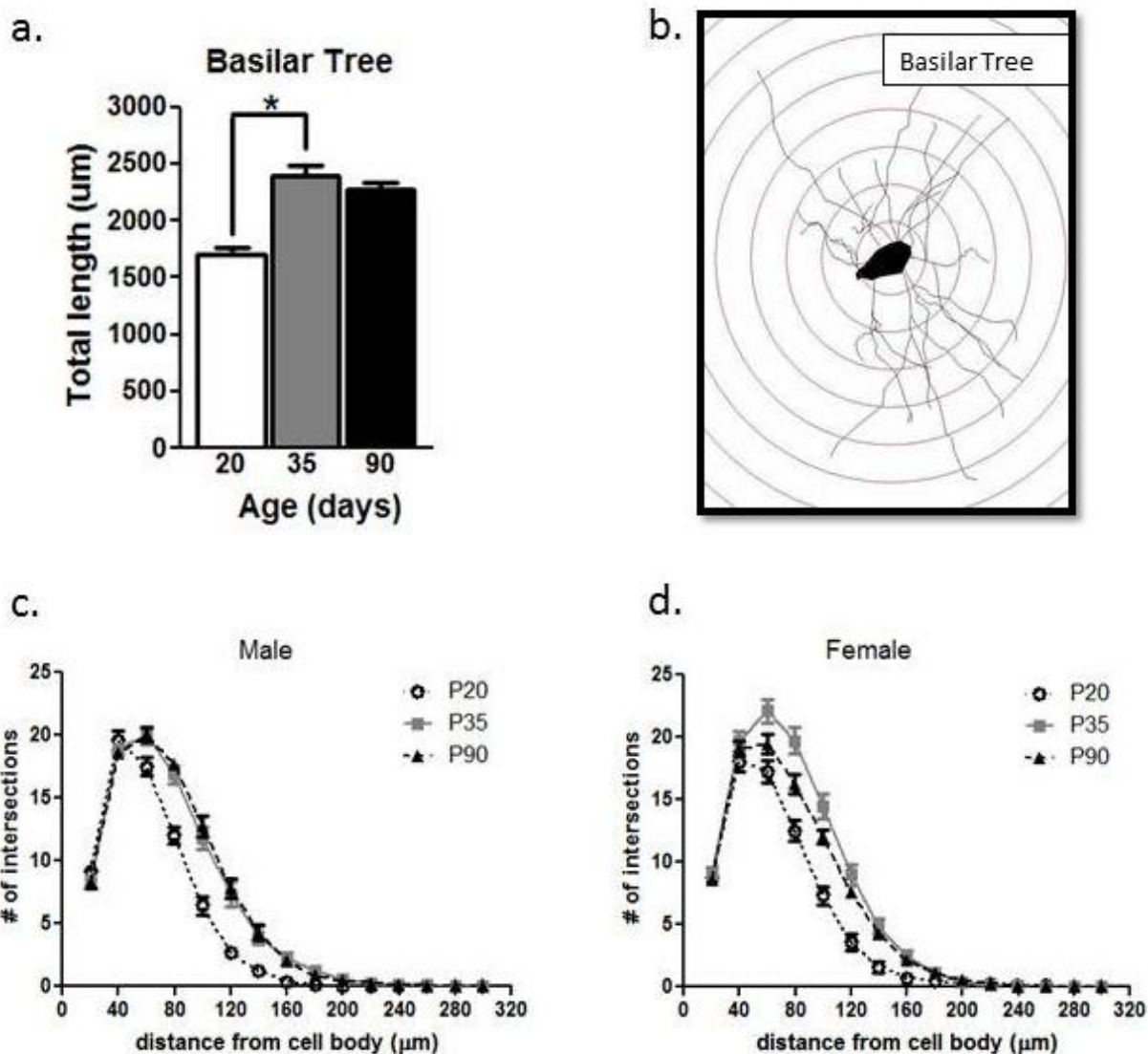
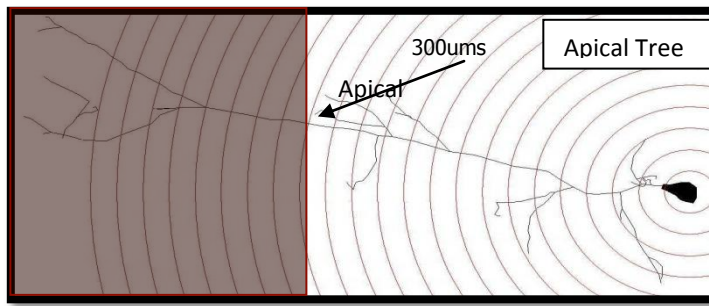
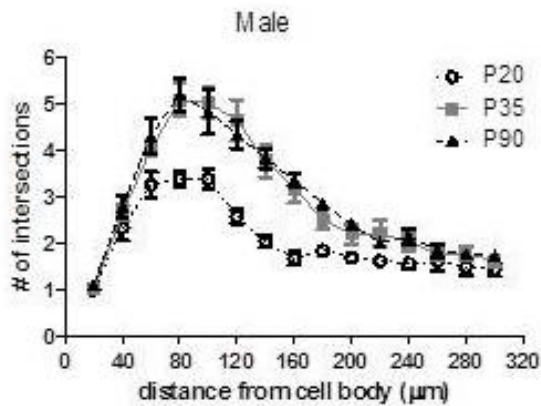


Figure 3.3 Dendritic branching analysis of the basilar tree of the mPFC. Total dendritic length (a), a representational drawing of the basilar tree (b), and male and female Sholl analysis (c). Between P20 and P35 dendritic growth was found in total dendritic length and in Sholl analysis in most of the basilar tree analyzed. From P35 to P90 there were no sex differences or changes in total length, but in the Sholl analysis a significant sex by order by age interaction was significant ( $p = 0.03$ ) indicating dendritic pruning during this time in females only. \* =  $p < 0.05$

a.



b.



c.

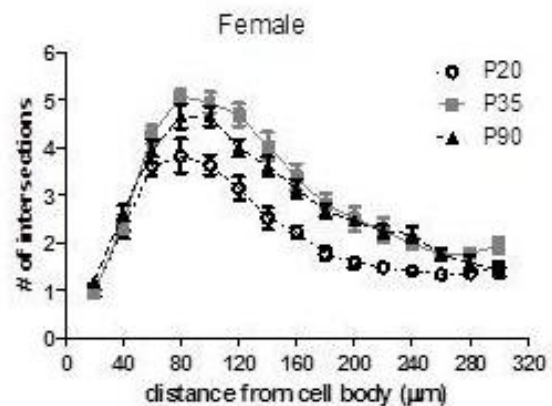


Figure 3.4 Dendritic analysis of apical oblique branches in the mPFC. A drawing of the apical region of a layer 5 pyramidal neuron (a) and subsequent results from Sholl analysis of apical obliques (b; within 300µm of the cell body). Data indicated large increases in dendritic material from P20 to P35 in both sexes in the majority of the apical dendritic tree analyzed. No differences were found between P35 and P90 and no sex differences were found.



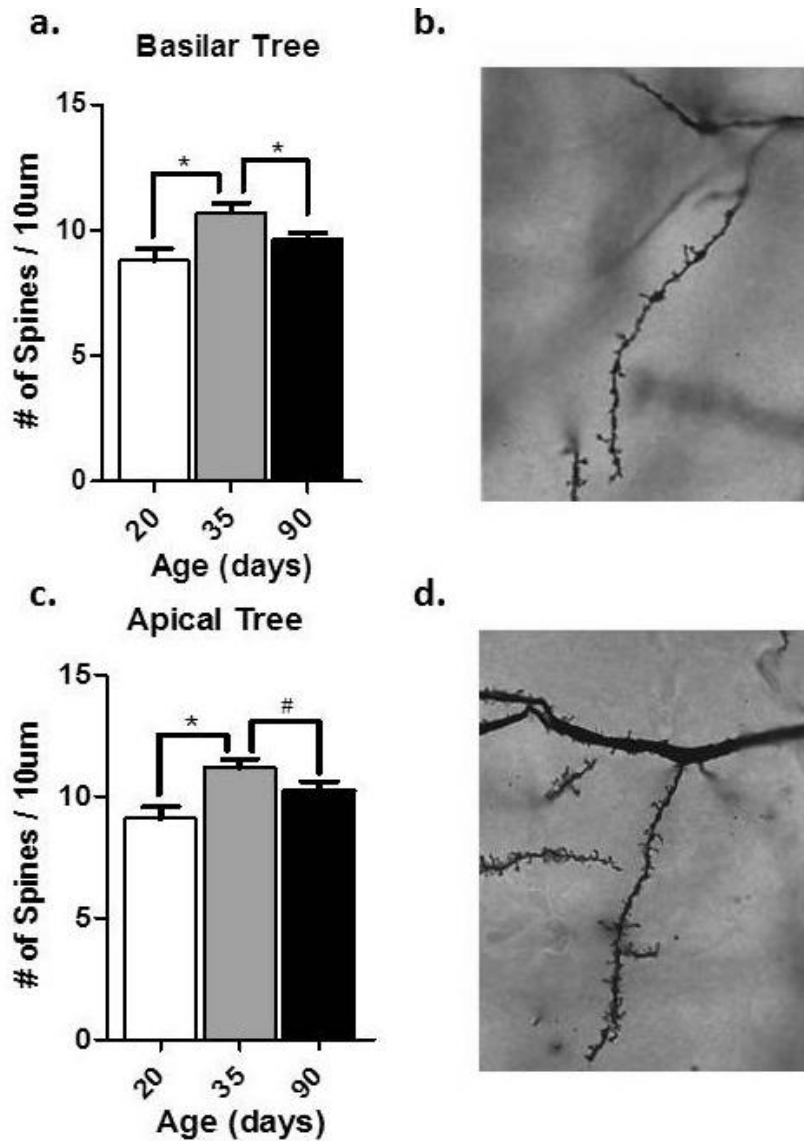


Figure 3.5 Dendritic spine density measures in the mPFC and photographic representations of Golgi filled dendritic spines for the PFC in the basilar tree (a-b) and in apical oblique branches (c-d). Measurements reported increases in both regions between P20 and P35 followed by a significant reduction in spines in the basilar tree and a trend for a decrease on apical oblique branches. \* =  $p < 0.05$ ; # =  $p < 0.08$

## **Chapter 4**

### **Adolescent Alcohol Exposure on the Neuroanatomy of the Medial Prefrontal Cortex and Basolateral Amygdala.<sup>2,3</sup>**

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<sup>2</sup> This chapter is an adaptation of a paper previously published entitled “Effects of ethanol during adolescence on the number of neurons and glia in the medial prefrontal cortex and basolateral amygdala of adult male and female rats” by Koss, W.A., Sadowski, R.N., Sherrill, L.K., Gulley, J.M., and Juraska, J.M (2012).

<sup>3</sup> The work performed in this chapter was performed in equal collaboration with Renee Sadowski.

## Introduction

Approximately 85% of people within the United States have had their first drink by the legal age of 21 (Grant and Dawson, 1997). Furthermore, binge-drinking rates steadily increase in adolescence from 7% to 34% between the ages of 14 and 20, a time period when heavy alcohol consumption is prevalent in both males and females (Johnston et al., 2008; Substance Abuse and Mental Health Services Administration, 2011). Given the prevalence of binge-drinking in humans during this time as well as reports of adolescent alcohol use increasing the susceptibility for future alcohol dependence (Grant and Dawson, 1997), it is important to evaluate the cellular changes associated with adolescent ethanol exposure in rodent models.

In adolescent rats, neural damage has been reported after exposure to high levels of ethanol. For example, ethanol compromises neurogenesis in the hippocampal dentate gyrus in adolescents to a much greater degree than in adults (Crews and Nixon, 2003; Crews et al., 2006). Silver staining, which is an indicator of cellular stress that may lead to cell death, immediately increases in the olfactory tubercle, hippocampal dentate gyrus, and the piriform, perirhinal, and entorhinal cortices after several days of high doses of ethanol administered during adolescence (Crews et al., 2000; Obernier et al., 2002). Obernier et al. (2002) showed evidence of neuronal death with Fluoro-Jade B, a pyknotic cell death marker, but not with the apoptotic marker, TUNEL, in the dentate gyrus. In addition, Pascual et al. (2007) found evidence of increased cell death through changes in DNA fragmentation and capsase-3 activity in the neocortex, hippocampus, and cerebellum of rats that had been exposed to high doses of ethanol from the juvenile through the adolescent period. This work makes it pertinent to investigate the long-term effects of adolescent ethanol exposure on the number of neurons in adults.

In addition to the hippocampus, the cerebral cortex and the amygdala may be particularly vulnerable to ethanol during adolescence and they are part of a circuit that is important for addiction. The BLN and the mPFC, in particular, receive dopamine innervations from the ventral tegmental area, a region that also supplies dopamine to the nucleus accumbens (Sesack et al., 2003). In humans, structural magnetic resonance imaging studies have shown that both the mPFC and BLN are continuing to develop throughout adolescence. The cerebral cortex decreases in volume during adolescence in a region- and sex-specific manner, with the largest decreases occurring in the prefrontal cortex (De Bellis et al., 2001; Giedd, 2004; Sowell et al., 1999). Conversely, the volume of the amygdala increases between childhood and adulthood with more change occurring in males than in females (Giedd et al., 1996a; Merke et al., 2003). The cellular basis for these changes has not been thoroughly investigated (Guillery, 2005), but there is evidence for a loss of synapses in the human and non-human primate mPFC (Anderson et al., 1995; Huttenlocher, 1979). In rats, our laboratory demonstrated multiple cellular alterations in both the mPFC and the BLN between the early adolescence period (P35) and adulthood (P90). In the mPFC and BLN, neuron number significantly decreases during this time period, whereas glia number decreases in the BLN of both sexes and in the mPFC a sex difference appears at day 90 due to a slight decrease of glia in females and an increase in males (Markham et al., 2007; Rubinow and Juraska, 2009). Additionally, spine density in the mPFC is decreasing in both sexes between adolescence and adulthood, but is unchanged in the BLN (Koss et al., 2009; Koss et al., 2010). At the same time, there are increases in BLN innervation to the mPFC (Cunningham et al., 2002), whereas mPFC efferents to the BLN are being pruned (Cressman et al., 2010). Given that these brain structures are undergoing such significant changes that are concurrent with the increase of ethanol consumption in humans, it is important

to investigate how ethanol alters these brain structures. Ethanol sharply increases naturally occurring neuronal death throughout the brain during the prenatal and early postnatal period in rats (Goodlett and Eilers, 1997; Ikonomidou et al., 2000; Miller and Potempa, 1990), including in the mPFC (Mihalick et al., 2001). The BLN is far less studied in the effects of ethanol; however ethanol has been shown to affect the glutamatergic and GABAergic neurotransmission in 4-6 week old rats in the BLN (Christian et al., 2012; Silberman et al., 2009), which may have consequences to long-term stress and anxiety levels. Furthermore, ethanol has effects on many types of glia including astrocytes and microglia (Evrard et al., 2006; McClain et al., 2011). The normal occurrence of neuronal death and alterations in the number of glia during adolescence may render the mPFC and BLN particularly vulnerable to alcohol exposure.

Work on the neural effects of alcohol exposure has been restricted to males, but there are indications that females might not exhibit the same effects. For example, humans display sex differences in the pattern of drinking and abuse (Greenfield and Rogers, 1999; Hasin et al., 2007; Witt, 2007) and in rats, sex differences appear in alcohol consumption during both adolescence and adulthood (Lancaster et al., 1996; Vetter-O'Hagen et al., 2009). Sex differences in consumption during adulthood also occur following binge-like exposure to alcohol during adolescence (Maldonado et al., 2008; Sherrill et al., 2011b), and these differences do not occur if females are ovariectomized before puberty (Sherrill et al., 2011b). We have also found sex differences in the effects of ethanol during adolescence on ethanol-induced conditioned taste aversion in adults (Sherrill et al., 2011a). Although few studies examine sex-dependent cellular changes in response to ethanol exposure during adolescence, binge-like exposure alters the expression of stress-related genes in the hypothalamus of male, but not female rats (Przybycien-Szymanska et al., 2010).

The present study examines the long-term effects of binge-like exposure to alcohol during adolescence on the number of neurons and glia in the rat mPFC and BLN. Both males and females were examined in light of sex differences in the response to alcohol, as well as the sex-specific development of the mPFC that we have previously demonstrated (Markham et al., 2007).

## **Methods**

### ***Subjects***

The male and female Long-Evans rats in this study were offspring of rats obtained from Simonsen Labs (Gilroy, CA) and bred in the Psychology department. Rats were maintained on a 12:12-h light-dark cycle (lights on at 0800h) with free access to food and water. At P25, pups were weaned and then double or triple-housed with same-sex littermates throughout the remainder of the experiment. Subjects came from three separate cohorts of rats, born 2-3 months apart. All animal procedures were in compliance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign.

### ***Ethanol Administration***

From P35-45, male (7 per group) and female (8 per group) rats received intraperitoneal (i.p.) injections of 3g/kg ethanol (25% v/v saline solution; Ricca Chemical, Arlington, TX) or saline. Injections were administered in a binge-like pattern, with one injection per day for 2 days followed by 1 day without an injection. This cycle was repeated 4 times so that 8 total injections of saline or ethanol occurred across a span of 11 days. This pattern of exposure has previously been utilized in our laboratory and in other studies that find behavioral or physiological effects of

adolescent ethanol exposure that persists into adulthood (Pascual et al., 2009; Philpot et al., 2009; Sherrill et al., 2011a; Sherrill et al., 2011b). BECs in alcohol-treated male rats were ~195mg/dl (Pascual et al., 2009). After completion of injections, rats were handled weekly until histology was performed in adulthood.

### ***Histology***

At P100, rats were weighed, injected (i.p) with 100 mg/kg sodium pentobarbital and then perfused intracardially with 0.1 M phosphate buffer saline (PBS) followed by 4% paraformaldehyde in PBS. After removal, a subset of the brains were weighed (males, n=5 per treatment; females, n=6 per treatment) and all brains were stored in the 4% paraformaldehyde solution for three weeks. Brains were then transferred to a 30% sucrose solution for 3 days, and then sectioned on a freezing microtome. Every fourth 60 µm section was mounted on slides and then stained with Methylene Blue/Azure II on the following day. All tissue was coded such that the experimenter was blind to group. Also, a single experimenter did all of the parcellations and cell counts on each neural area to maintain consistency.

### ***Volume***

Parcellations of both the ventral mPFC (infralimbic and prelimbic regions) and the BLN were performed based on differences in cytoarchitecture as described previously (Krettek and Price, 1978; Markham et al., 2007; McDonald, 1982; McDonald et al, 1996; Rubinow and Juraska, 2009; Van Eden and Uylings, 1985) (Figures 4.1). Parcellations of the boundaries of the mPFC were conducted from the most anterior mounted section where the underlying white matter appeared and continued on every mounted section until the appearance of the genu of the corpus callosum, resulting in analysis of 4-6 sections for each brain. Within each parcellation, dorsal and ventral boundaries were drawn as well as for the upper layers 2/3 and lower layers 5/6

(the rat mPFC does not have a layer 4). Layer 1 was not included because it contains very few cells. Duplicate drawings of boundaries were done in a subset of animals to confirm consistency within 5% of the original parcellations. The entire extent of the BLN (10-11 sections per brain) was parcellated separating it from the multiple nuclei in the basolateral complex (lateral, basomedial, and basolateral ventral amygdala). In both brain structures, areas were obtained and post-shrinkage thickness was measured during cell counting and used to determine an average thickness. Volumes were calculated with the Cavalieri method (Mouton, 2002) as the product of the areas and the measured tissue thickness between the saved sections. In the mPFC, all of these measurements were calculated for each layer as well as all layers combined.

### ***Neuron and Glia Number***

Total number of neurons and glia were determined using the optical dissector with the Stereoinvestigator program (Microbrightfield; Williston, VT), as described previously (Markham et al., 2007; Rubinow and Juraska, 2009). For both neuron and glia density, the entire anterior-posterior extent of the mPFC and the BLN was quantified including separate quantifications for the upper and lower layers of the mPFC. The computer program chose the location of counting frames, which measured 35 $\mu$ m x 35 $\mu$ m (length x width), within parcellated boundaries. Guard zones were set at 1 $\mu$ m for the mPFC and 1.5 $\mu$ m for the BLN at the top and bottom of each section with both brain regions utilizing a dissector height of 13 $\mu$ m. A cell was counted only if the bottom of the cell was within the volume of the counting frame. Both neurons and glia were separately counted within the frame. They were distinguished based on differences in morphological, size, and color characteristics as described by Markham et al. (2007) and Rubinow and Juraska (2009) (Figure 4.2). At least 200 neurons and 140 glia were counted from the BLN and from the upper and lower layers of the mPFC for each animal. These numbers



were divided by the total volume of the counting frames to determine neuron and glia density. The densities for each animal were then multiplied by the volume of the structure for that animal to determine total number of neurons and glia. In the mPFC, this was done for the upper and lower layers separately as well as the all layers combined

### ***Statistical Analysis***

Two-way analysis of variance (ANOVA), using sex and treatment as factors and cohort as a covariate, was performed for all analyses. Post-hoc tests were performed using one-way ANOVAs of treatment or sex effects so that the cohort covariate could be used.

## **Results**

### ***Body and Brain Weights***

As previously reported in our laboratory (Sherrill et al., 2011b), at the last day of injection (P45), ethanol administration significantly decreased body weight in males and females ( $F(1,27)=8.0$ ,  $p=0.009$ ); however this effect was not permanent (Table 4.1). All animals compensated for this weight loss and were not significantly different at P100. There were significant sex differences in body weights at both ages with males weighing more than females (P45,  $F(1,27)=24.1$ ,  $p<0.001$ ; P100,  $F(1,28)=74.0$ ,  $p<0.001$ ). Brain weights also differed between the sexes with males being greater ( $F(1,17)=16.6$ ,  $p=0.001$ ), but ethanol treatment had no effect on brain weight (Table 4.1).

### ***Medial Prefrontal Cortex***

An example of a mPFC parcellation used to quantify volume is shown in Figure 4.1a. Ethanol administration had no detectable effect on the volume of the whole mPFC or on individual layers. There was a weak trend ( $F(1,25)=3.1$ ,  $p=0.09$ ) towards a sex difference in

volume, with females having a smaller volume than males. Neurons and glia were separately counted (Figure 4.3), and there was a trend towards a sex difference ( $F(1,25)=3.7$ ,  $p=0.07$ ) in total number of neurons in all layers, with females having 10% fewer neurons than males (Figure 4.3a), similar to previous results (Markham et al., 2007). The sex difference reached significance in the upper layers (Figure 4.3b;  $F(1,25)=5.1$ ,  $p=0.034$ ), but not in the lower layers (Figure 4.3c). There were no significant effects of treatment and no treatment by sex interaction for neuron number in all layers combined or in individual layers (Figure 4.3).

Analysis of glia number across all layers resulted in a strong trend ( $F(1,25)=4.1$ ,  $p=0.053$ ) towards a sex by ethanol-treatment interaction. Further analysis revealed that this interaction was based on a significant decrease ( $F(1,11)=8.0$ ,  $p=0.02$ ) of 14% in male rats that received ethanol compared to those that received saline, while no difference occurred between treatment groups in females (Figure 4.3d). This pattern was present in both the upper ( $F(1,11)=5.6$ ,  $p=0.04$ ) and lower ( $F(1,11)=7.2$ ,  $p=0.02$ ) layers with males, but not females, showing differences between treatments (Figure 4.3e and 4.3f). As demonstrated previously by our lab (Markham et al., 2007), a trend towards a lower number of glia was found in female controls compared to male controls in combined layers ( $F(1,12)=4.1$ ,  $p=0.07$ ) and in the lower layers ( $F(1,12)=4.6$ ,  $p=0.053$ ).

### ***Basolateral Nucleus of the Amygdala***

A representation of a BLN parcellation used to quantify volume is shown in Figure 4.1b. Neither sex nor ethanol treatment had a significant effect on volume of the BLN. The lack of sex difference in controls replicates our prior findings in the BLN reported by Rubinow and Juraska (2009). Similar to the mPFC, total neuron number was not significantly affected by alcohol (Figure 4.4a). As previously found there was also no sex difference in neuron number in the

BLN (Rubinow and Juraska, 2009). In contrast to the mPFC, there was no significant treatment effect in total glia number in the BLN (Figure 4.4b). There also was no sex difference in glia number confirming prior results (Rubinow and Juraska, 2009).

## **Discussion**

The present study showed that repeated binge-like exposure to alcohol during adolescence did not alter the number of neurons in the mPFC or the BLN in adulthood in either male or female rats. There was, however, an effect of ethanol exposure on glia number in the mPFC, but not in the BLN. In the mPFC, ethanol-treated males had fewer glial cells in comparison to controls while females were unchanged. Given the interval between the last dose of ethanol (P45) and the time the brains were collected (P100), these differences are not due to the immediate effects of alcohol exposure but are instead long-term, and perhaps permanent, changes in the brain.

While the lack of effects of ethanol on neuron number was not predicted given the neuronal losses normally occurring in the mPFC and the BLN during adolescence (Markham et al., 2007; Rubinow and Juraska, 2009), there is support for the observed effects of ethanol on glia in the mPFC. Human alcoholics display a substantial decrease in glia density in the prefrontal cortex (Miguel-Hidalgo et al., 2002; Miguel-Hidalgo et al., 2006b) and glia number in the hippocampus (Korbo, 1999). Furthermore, in one of these studies neuron number was not altered (Korbo, 1999) but in another study neuronal density was decreased (Miguel-Hidalgo et al., 2006b). In rodent models, ethanol impedes glia proliferation in cell cultures (e.g., Guerri et al., 1990; Kennedy and Mukerji, 1986), and prenatal exposure results in fewer glia cells (but also neurons) in adult somatosensory cortex (Miller and Potempa, 1990). Unfortunately, the

neural effects of exposure to ethanol during adolescence in rats are limited. One report has found a short-term decrease in glia density in the hippocampus (Oliveira-da-Silva et al., 2010) which is consistent with the current study. However, in the frontal cortex a decrease in cytoplasmic optic density of S100 $\beta$  immunoreactive astrocytes was found along with an increase in GFAP-immunoreactivity within astrocytes immediately after ethanol administration (Evrard et al., 2006). Also in the same study it was reported that after 10 weeks both levels either returned to control levels (S100 $\beta$ ) or trended closer to the level of controls (GFAP-ir) (Evrard et al., 2006). However, how both of these measures relate to the number of glia rather than changes in expression of the marker is unknown. An additional study has found no immediate change in GFAP immunoreactive cell density but an increase after a 3-day withdrawal in alcohol-preferring rats (Miguel-Hidalgo, 2006a). Comparing this study with the current study is also difficult because of the differences in the strain of rat, the timing of withdrawal, and the exposure of ethanol differed greatly (11 days in the current study versus 2 months in the previous study).

The decrease in glia in the male mPFC can be a result of either an increase in cell death or a decrease in proliferation. There are indications that ethanol interferes more potently with proliferative processes than cell death once the organism is beyond early development (Miller, 2003). For example, in the hippocampal dentate gyrus, exposure to ethanol decreases the proliferation of neurons that is normally observed during adulthood (Nixon and Crews, 2002), and this effect is even more severe in adolescence (Crews et al., 2006). Likewise, the subventricular cells that give rise to the olfactory granule neurons, which proliferate throughout life, are decreased by ethanol exposure (Hansson et al., 2010). Effects of ethanol on proliferation would have made glia in the mPFC more vulnerable than neurons, which are post-mitotic after birth. More specifically, ethanol blocks a mitogenic growth factor (PDGF), which regulates

astrocytic proliferation *in vitro* (Luo and Miller, 1999). In the mPFC, Markham et al. (2007) found no sex differences in the number of glia at P35, the first day of ethanol exposure in the present study, but males had significantly more glia than females by adulthood (P90) because the number of glia increased in males between these ages, while the absolute number of glia decreased in females. Therefore, ethanol exposure appears to have interfered with the developmental trajectory of proliferation of glia that occurs specifically in males. In contrast, glia number in the BLN decreased in both sexes between the adolescence and adulthood (Rubinow and Juraska, 2009), indicating that cell death of glia in the BLN may be greater than the proliferation of glia, and that proliferation in the BLN is attenuated compared to the mPFC. Thus, the differential effects of ethanol on glia in the mPFC and BLN may reflect contrasting proliferation rates of glia between these two brain regions.

The changing properties of neurons and their receptors during development may account for the present finding that ethanol exposure during adolescence did not cause neuronal death in the mPFC or BLN as it does early in development (Ikonomidou et al., 2000; Ikonomidou et al., 2001). It has been suggested that both the NMDA antagonist and GABA<sub>A</sub> agonist properties of ethanol are responsible for triggering cell death (Olney et al., 2002). Likewise, there is widespread attenuation of many cortical effects of prenatal ethanol in NMDA-NR1 knockout mice (Deng and Elberger, 2003). Both glutamate and GABA<sub>A</sub> receptors change in their properties and number between the early neonatal period and adolescence, which may alter the apoptotic response to ethanol or the degree of this response (Insel et al., 1990; Wang and Gao, 2010; Yu et al., 2006).

Although sex differences in ethanol pharmacokinetics are well established, it is unlikely that similar sex differences in adolescents are the source of sex differences that we observed in

mPFC anatomy. Notably, while females have been shown to exhibit faster blood ethanol elimination rates than males, it has also been demonstrated that there are no difference in pharmacokinetic profiles for brain ethanol concentrations (BrECs; Crippens et al., 1999). Moreover, a recent study found that although BrEC and blood ethanol concentrations (BECs) differed between adult male and female animals given 2 g/kg ethanol, but neither BrEC nor BECs differed in adolescents (Morales et al, 2011). In our previous study, which utilized the same adolescent treatment procedure, we found that intoxication ratings were not significantly different between male and female rats (Sherrill et al., 2011b). Thus, although we did not measure BrECs or BECs in the present study and therefore cannot definitively determine the role of metabolism in our findings, it is unlikely that this would explain the reductions in mPFC glia in ethanol-exposed males, but not females.

What is known about the neural effects of ethanol is predominantly based on male animals, but glia both manufacture and respond to the gonadal steroids (Garcia-Segura and Melcangi, 2006), which complicates the effects of ethanol on cell death. Furthermore, it should be noted that the time of ethanol exposure in the current study coincides with the appearance of puberty markers in our vivarium (female vaginal opening = ~day 36; male pubertal separation = ~42; Koss et al, unpublished data). Estrogen is known to decrease cell death in adult rodents following hypoxia-ischemia (Wise and Dubal, 2000; Zhu et al., 2006). Since cell death markers do not discriminate between neurons and glia, estrogen may decrease the probabilities of glia death with exposure to ischemia or potentially, ethanol. Interestingly, there is also less cell death in organotypic cultures derived from females, compared to males, in response to oxygen and glucose deprivation as well as NMDA excitation (Li et al., 2005). These findings have led to the speculation that in addition to its activational role, estrogen may also have organizational or

epigenetic effects that do not require estrogen to be present for its protective influence (Siegel et al., 2010). Obviously, more work is needed on the role of estrogen and other gonadal steroids in influencing the effects of ethanol.

In the current study we observed a significant sex difference in the number of neurons in the upper layers of the mPFC, but unlike Markham et al (2007), we did not demonstrate this when all layers were combined. There were also only trends in overall volume or number of glia. This could be due to increased variability in the present study caused by injection stress during adolescence (Brown et al., 2005) which did not occur in the Markham et al study. The present study also had a smaller sample size of the control animals, and in Markham et al (2007) litter was represented across groups so that litter was used as a factor to decrease variance. Both studies showed similar percent difference between the sexes in the total number of neurons (10% in the present study versus 13%), the total number of glia (20% versus 18%) and volume (13% versus 18%).

Determining the types of glia that were affected in the male mPFC in the current study should be the next step in understanding this effect of ethanol. The cortex contains the major classes of glia, including astrocytes, oligodendrocytes, and microglia. In the current study, astrocytes are one of the likely classes of glia that were decreased in the male mPFC after ethanol exposure. Previous work has shown acute increases in GFAP immunoreactivity with adult or prenatal ethanol exposure, but in the long-term these increases are significantly reduced or not detectable (Franke et al., 1997; Goodlett et al., 1993; Rintala et al., 2001). Also, in cortical cultures, ethanol initiates astrocytic cell death (Blanco et al., 2005; Kane et al., 1996). Additionally, male oligodendrocytes may also be affected by ethanol exposure in adolescence. During normal development of the cortex in male rats, the number of oligodendrocytes, as

opposed to astrocytes and microglia, increase dramatically between 1 month and 3.5 months of age in the rat cortex (Ling and Leblond, 1973). Therefore, ethanol may decrease oligodendrocyte proliferation, thereby preventing the increase in glia number that occurs only in males during adolescence (Markham et al., 2007). Opposite to the decrease in glia seen in the cortex of the current study, microglia in males tend to increase in response to ethanol in both the cerebellum of adult rats (Riikonen et al., 2002) and in the hippocampus of adolescent rats (McClain et al., 2011). These studies may help further explain why there was no effect of ethanol on glia in the BLN. The BLN may be comprised of different proportions of subtypes of glia compared to the mPFC. For instance, if the BLN contains more microglia than the mPFC, that would negate the decrease in astrocytes or oligodendrocytes. To date, this kind of comprehensive study of glia cell type proportions in either of these brain regions is unavailable, thus making it difficult to predict which glia cell type is being altered by alcohol. However, it is unlikely that changes in microglia contribute to the decreases of glia seen in the mPFC in the current study. Regardless, the types of glia that are decreased following adolescent ethanol exposure in males needs to be directly established.

The decrease in the number of glia in the mPFC in males could have long-term consequences for neural function and behavior. Glia, and astrocytes in particular, are involved in regulating the extracellular chemical environment, supplying energy to neurons, and participating in several aspects of neuroprotection, such as the production of neurotrophic factors and the homeostasis of neuronal glutathione (Gonzalez and Salido, 2009; Watts et al., 2005). A decrease in the number of glia may result in slower neural processing when mPFC functions are taxed, and long-term effects may include less protection from drugs that induce oxidative stress, including ethanol itself (Allaman et al., 2011; Watts et al., 2005). Furthermore, even though we



found no changes in the BLN after adolescent ethanol exposure, future studies may find changes within specific glia types. Clearly, more work is needed to understand how a decrease in glia may alter other changes occurring during adolescence in males and the mechanism by which the sexes differ in their response to ethanol during adolescence.

## Tables

Table 4.1 Body and Brain Weights

	P45 Body Weight (mean grams $\pm$ SEM)	P100 Body Weight (mean grams $\pm$ SEM)	P100 Brain Weight (mean grams $\pm$ SEM)
Males			
Vehicle	176.1 $\pm$ 7.7	375.0 $\pm$ 21.4	1.47 $\pm$ 0.04
ETOH	154.0 $\pm$ 4.8	388.6 $\pm$ 12.1	1.45 $\pm$ 0.01
Females			
Vehicle	143.7 $\pm$ 2.0	257.4 $\pm$ 3.8	1.38 $\pm$ 0.01
Ethanol	138.8 $\pm$ 2.8	266.3 $\pm$ 5.0	1.34 $\pm$ 0.03

At P45 the last day of injections, ETOH treated animals weighed significantly less regardless of sex ( $p=0.009$ ). There were no differences due to treatment later in adulthood (P100). Adult brain weights have a sex difference ( $p=0.001$ ) but no ETOH effects.

## Figures

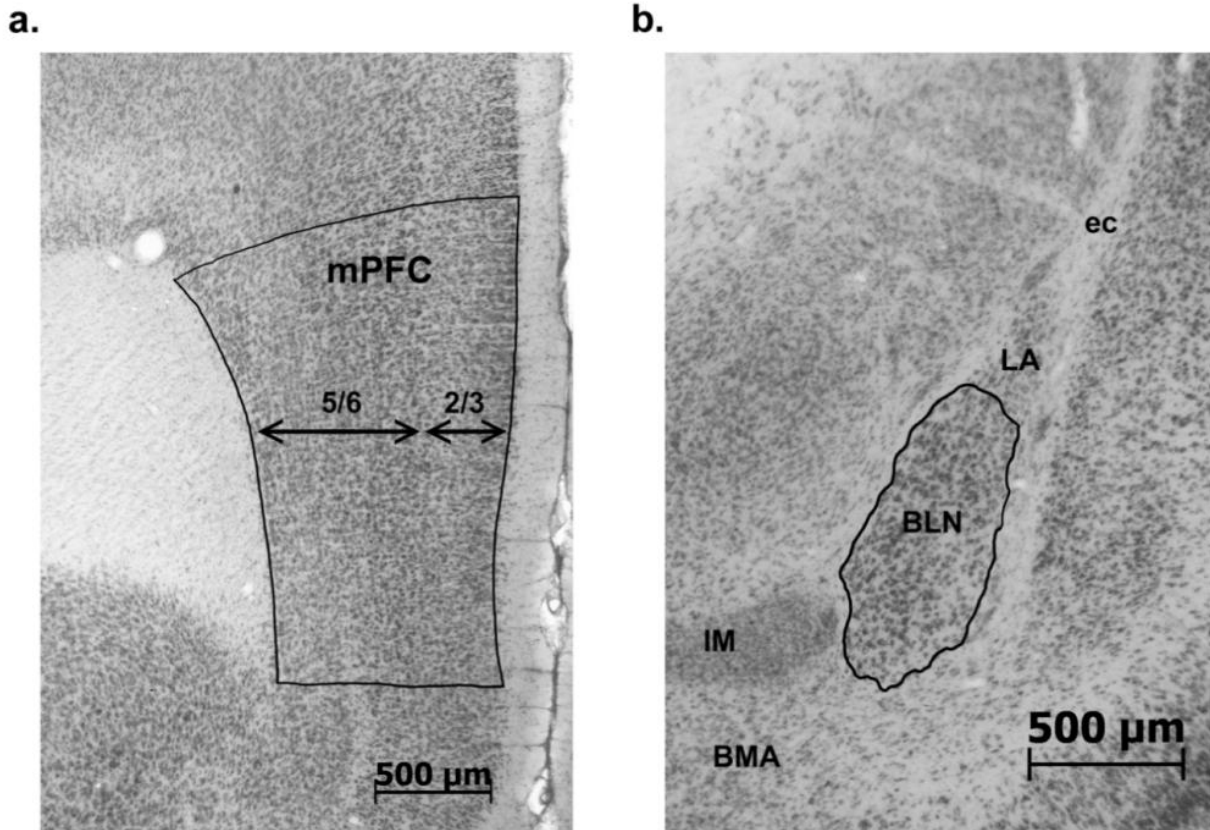


Figure 4.1 A photograph of the mPFC (a) and the anterior BLN (b). In the mPFC, layers 2/3 and 5/6 are distinguished, and in the BLN white matter and surrounding nuclei are labeled. LA = Lateral Amygdala; BMA = Basomedial Amygdala; ec = external capsule; IM = main intercalated nucleus

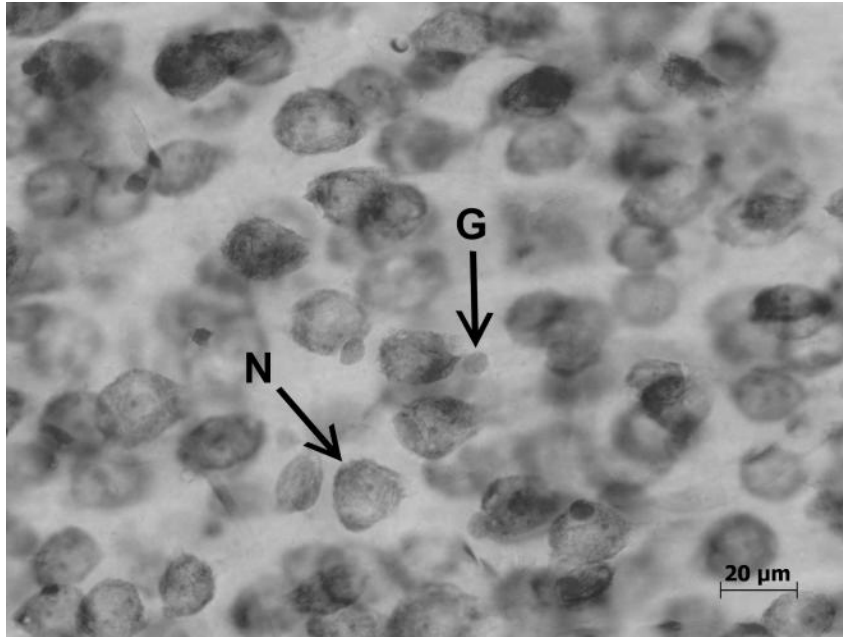


Figure 4.2A photograph illustrating a neuron (N) and glia cell (G) at the high magnification used for counting. Note the distinguishing characteristics in size and shape.

## mPFC

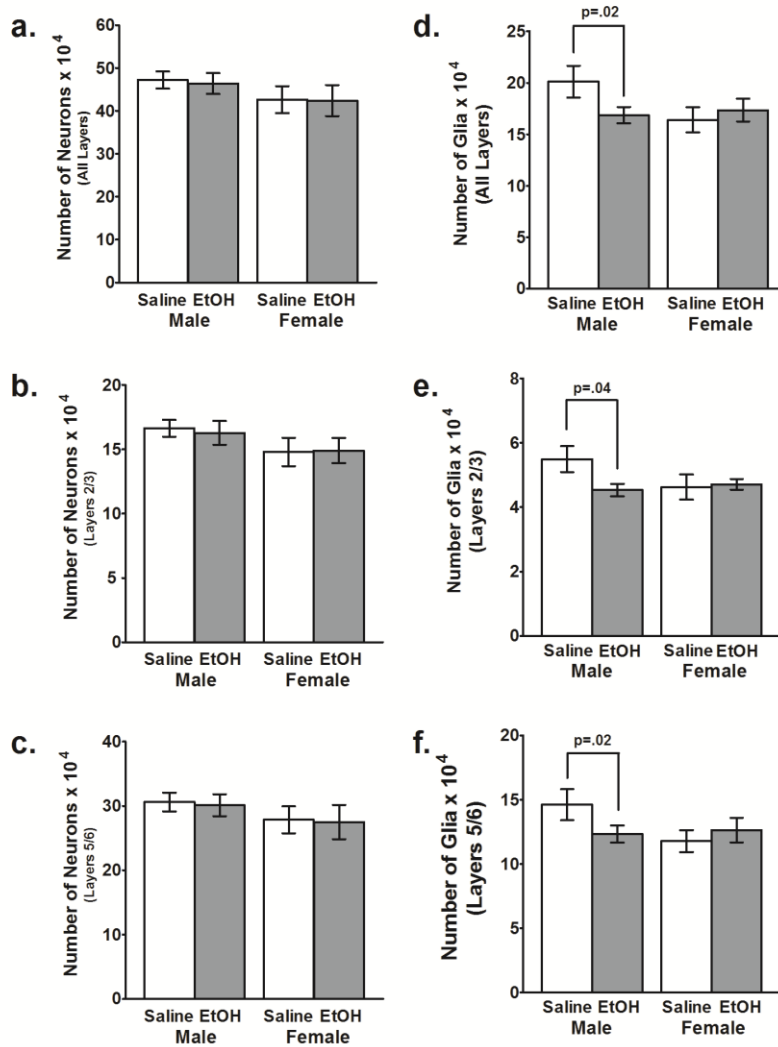


Figure 4.3 The number of neurons (mean + SEM) in adults in all layers (a), layers 2/3 (b), and layers 5/6 (c) of the mPFC. No effects of ethanol on neuron number were found, but a significant sex difference was present in layers 2/3 whereas only a trend was found in layers 5/6 and combined layers. The number of glia (mean + SEM) is also shown in all layers (d), layers 2/3 (e), and layers 5/6 (f). There was a significant effect of ethanol on glia in males only in separate and combined layers. There was also a trend for a sex difference in the controls in combined layers and layers 5/6.

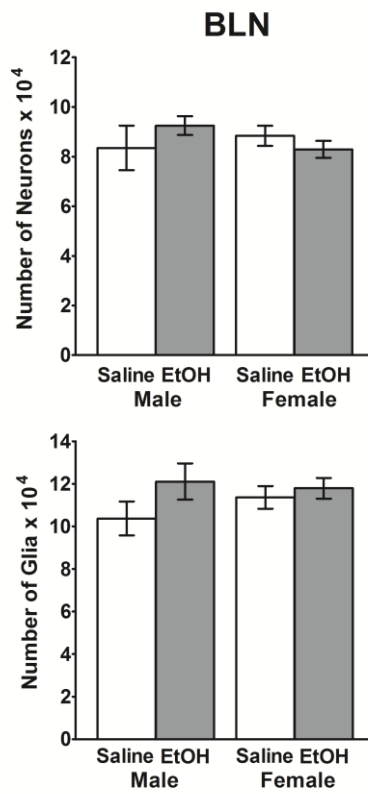


Figure 4.4 The number of neurons (a) and glia (b) in the adult BLN (mean + SEM). There were no significant sex or treatment differences in the BLN.

## **Chapter 5**

### **Effects of Prepubertal Gonadectomy on the Number of Neurons in the Medial Prefrontal Cortex**

## Introduction

Testicular androgens have profound effects on sex differences during early development especially in neural areas associated with reproduction (MacLusky et al, 1994; Gorski et al, 1978; Arai et al, 1996; Tsukahara, 2009). More recently, both androgens and ovarian hormones have been found to organize several reproductive and non-reproductive brain areas during puberty in a sex-specific manner by attenuating or triggering apoptosis, as well as by increasing cell genesis. For instance, the anteroventral periventricular nucleus (AVPV), a structure that is larger in adult females than adult males, does not differ from males until after postnatal day (P) 30 (Davis, 1996). Additional work has shown that new neurons and glia are being added to the female AVPV at puberty and if the ovaries are removed prior to puberty both neurogenesis and gliogenesis is attenuated (Ahmed et al, 2008). Furthermore, the sexually dimorphic nucleus of the preoptic area (SDN) and the medial amygdala, which are larger in adult males than females, also appears not to fully develop until after puberty. In both of these neural regions pubertal castration eliminates the sex difference in cell number and volume (Ahmed et al, 2008). The only region in the cerebral cortex where effects of pubertal hormones has been directly examined is the visual cortex where adult males have more neurons than females (Reid and Juraska, 1992; Nunez et al, 2002). Work from our laboratory found that the removal of the ovaries prior to puberty resulted in more neurons in adult female rats and eliminated the sex difference that appears in adulthood (Nunez et al, 2002). In the same study, removal of the testes in males had no effect on the number of neurons. Ovarian hormones released during puberty have also been implicated in decreased myelination in the corpus callosum (Yates and Juraska, 2008b) and dendritic spine losses in the visual cortex (Munoz-Cueto et al, 1990), whereas pubertal androgens have been associated with dendritic losses in the hippocampus (Meyer et al, 1978).



Like the visual cortex, the rat medial prefrontal cortex (mPFC) is sexually dimorphic. Adult males have a larger volume, more neurons and more glia than females (Markham et al, 2007). This sex difference does not appear until after adolescence. At this time there is a loss of neurons in both sexes but it is greater in females (5% males vs. 19% females; Markham et al, 2007). This is similar to human imaging studies where the gray matter volume of the prefrontal cortex declines during adolescence (Giedd et al, 1996b; Sowell et al, 1999; Jernigan et al, 1991). This decline begins 1-2 years earlier in females than males (Giedd et al, 1996b) and although not yet established, these differences could be due to the earlier onset of puberty in females and/or the release of gonadal hormones.

The current study tests the hypothesis that gonadal steroids during puberty alter the number of neurons and glia in the mPFC. We hypothesize that the mPFC is similar to the visual cortex where ovarian hormones are playing a role in the neuroanatomical organization of the structure by inducing cell death during puberty. In contrast, steroids coming from the testes, androgens, will have little effect on cell number in neurons or glia. This will be tested by performing gonadectomy and sham (control) surgeries on male and female rats prior to puberty followed by stereologically counting of neurons and glia in adulthood.

## **Methods**

### ***Subjects***

All rats were Long Evans male and female offspring from breeders obtained from Harlan (Indianapolis, IN). Rats were maintained on a 12:12-h light-dark cycle (lights on at 0800h) with free access to food and water. Surgeries were performed on animals at P20-22, with P0 being the day of birth. Experimental animals had their ovaries or testes removed and for each

experimental animal there was a same-sex, litter-matched control that experienced a sham surgery as well as equal amounts of anesthesia and handling. At P25, pups were weaned and housed with sex- and litter-matched control throughout the remainder of the experiment. Subjects came from two separate cohorts of rats, born 2 months apart with different breeding pairs. All animal procedures were in compliance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign.

### ***Gonadectomy Surgeries***

Surgeries were performed, one litter per day, similar to those done previously in our laboratory (Yates and Juraska, 2008b; Nunez et al, 2002). On P20-22 animals were separated from their mother and temporarily housed together in one separate cage. Each gonadectomy and sham surgery was completed using 2.75% isoflurane gas anesthesia with an average duration period of 45 minutes. Both ovariectomies and castrations were performed with a ventral incision in the lower abdominal area through the skin and then another through the muscle wall, after which testes or ovaries were located on each side of the rat, tied off with absorbable surgical thread and removed. The muscle incision, followed by the skin incision, was closed using sutures and animals were then placed in a separate cage until fully recovered from anesthesia. During surgeries, pups were removed from their mothers for a maximum of 6 hours. Post-surgical checks were performed for 7 days which involved a small amount of handling. Sutures were removed 7 days after surgery, if they had not fallen out before that time.

### ***Puberty Markers***

Starting at P28, rats were handled, weighed and checked for the appearance of puberty markers daily until P45 or until puberty markers were apparent. Vaginal opening was used as a

puberty marker in females, which has been demonstrated to coincide with increased estrogen levels and the beginning of LH surges in the pituitary (Castellano et al, 2011). In males preputial separation was used as a marker of puberty and has been shown to be a marker of the beginning of significant androgen secretion (Korenbrod et al, 1977). Vaginal opening and preputial separation never occurred for ovariectomized (OVX) females and castrated (CST) males. In intact animals, vaginal opening occurred between P33-P39 and preputial separation occurred between P40-45 except for one male where puberty onset was at P51.

### ***Histology***

At P90-91, rats were weighed, injected (i.p) with 100 mg/kg sodium pentobarbital and perfused intracardially with 0.1 M phosphate buffer saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were removed, weighed and stored in 4% paraformaldehyde solution overnight, then transferred to a 30% sucrose solution for 4 days until sectioning. Sixty micron sections were cut using a freezing microtome and every 4<sup>th</sup> section was mounted on slides and stained with Methylene Blue/Azure II the following day.

### ***Volume and Cell Number Quantification***

Parcellations of the ventral mPFC (infralimbic and prelimbic regions) were performed based on differences in cytoarchitecture as described previously in chapter 4 (Figure 4.1) and Markham et al. (2007). Parcellations of the boundaries of the mPFC were taken from the most anterior mounted section in which the white matter appeared and were continued through every mounted section until the appearance of the genu of the corpus callosum. This resulted in the analysis of 4-6 sections for each brain. Within each parcellation, dorsal and ventral boundaries were drawn as well as the boundaries for upper layers 2/3 and lower layers 5/6 (the rat mPFC does not have a layer 4). Layer 1 was not included because it contains very few neurons.

Volumes were then calculated with the Cavalieri method (Mouton, 2002) as the product of the areas of each section, the post-shrinkage tissue thickness and the number of sections between each saved section. Due to dissimilarities in the amount of cellular loss between the sexes in the different layers of the mPFC found by Markham et al (2007), all measurements were recalculated separately for upper layers (layers 2 and 3), lower layers (layers 5 and 6) as well as the entire mPFC (all layers).

Total number of neurons and glia were determined using the optical disector with the StereoInvestigator program (MicroBrightfield; Williston, VT), as described previously in chapter 4 (Figure 4.2) and others from our laboratory (Nunez et al, 2002; Markham et al., 2007; Rubinow and Juraska, 2009). Neuron and glia density were measured from both hemispheres throughout the entire anterior-posterior extent of the mPFC. The counting frame used was 35  $\mu$ m x 35  $\mu$ m x 15  $\mu$ m (length x width x height) with 1.5  $\mu$ m guard zones. A cell was counted only if the top of the cell was within the volume of the counting frame. Neurons and glia were separately counted within the frame and were distinguished based on differences in morphology, size, and color as described in chapter 4. At least 200 neurons and 100 glia were counted from each of the upper and lower layers of the mPFC for each subject. These numbers were divided by the total volume of the counting frames to determine neuron and glia density and then multiplied by the total volume of the structure to determine the total number of neurons and glia.

### ***Statistical Analyses***

Pre-planned *t* tests comparisons were used for all measures (Hays, 1994). Comparison analyses were performed between same-sex sham and gonadectomized groups (i.e. male GDX versus male sham and female GDX versus female sham) to test the effects of pubertal hormones. Each sham control, or intact animal, was paired with a GDX animal of the same sex, from the

same litter and was exposed to anesthesia for the same amount of time. Additionally both sham control groups were compared to identify sex differences.

## **Results**

### ***Body and Brain Weights***

Body weight was altered by gonadectomy in both sexes. The removal of the ovaries in females and the testes in males significantly altered body weight (Table 5.1). Male sham animals weighed 20% more than castrated males ( $p < 0.0001$ ) and female control animals weighed 31% less than OVX female rats ( $p < 0.0001$ ). This effect has been previously reported from our laboratory (Sherrill et al, 2011b; Yates and Juraska, 2008b; Nunez et al, 2002) and others (Wade, 1975; Slob et al, 1975). Additionally, intact males also had greater body weights than intact female rats ( $p < 0.0001$ ).

There were no differences in brain weight between the male groups in brain weight. In females, sham animals had lower brain weights (6%) than female OVX animals ( $p < 0.05$ ; Table 5.1), which had appeared as statistical trends in prior studies from our laboratory (Yates and Juraska, 2008b; Nunez et al, 2002). Also the brain weight of the intact males was greater than intact females ( $p < 0.01$ ).

### ***Volume and Cellular Counts***

Analysis of the combined upper (2/3) and lower (5/6) layers of the mPFC revealed that OVX females had a larger mPFC volume ( $p < 0.01$ ; Figure 5.1c), greater numbers of neurons ( $p < 0.05$ ; Figure 5.1a) and more glia ( $p < 0.01$ ; Figure 5.1b) than sham controls. Castrated males were not significantly different than sham control males on any measure (Figure 5.1), and

control males had a larger volume ( $p < 0.04$ ) and more glia ( $p < 0.02$ ), but not more neurons than control females.

When the upper layers and lower layers were separately analyzed, effects in the lower layers (5/6) were found to account for the majority of the differences seen in the entire mPFC. In layers 5/6, volumes were larger in OVX females when compared to sham controls ( $p < 0.01$ ). Total number of neurons ( $p < 0.05$ , Figure 5.2d) and glia ( $p < 0.01$ , Figure 5.2e) in layers 5/6 were also both significantly higher in OVX females than in control females. In controls, males had a larger volume ( $p < 0.02$ ), greater number of neurons ( $p < 0.05$ ), and a trend for more glia ( $p < 0.07$ ) than females. In layers 2/3, the volume was larger in OVX females than intact females ( $p < 0.05$ ; Figure 5.2c), but the number of neurons and glia were not significantly different (Figure 5.2a-b). As seen in the overall measures there were no volume, neuron or glia number differences between males who had their testes removed before puberty and intact males (Figure 5.2a-c). There were also no significant sex differences seen in any of these measures in the upper layers of the mPFC.

## **Discussion**

The present study found that females that had their ovaries removed before puberty had more neurons in the mPFC than control females. These results suggest the involvement of ovarian pubertal hormones, not androgens, are responsible for the neuronal loss during adolescence in the mPFC. The effect was restricted to layers 5/6 and did not occur in males after castration. This is in agreement with prior results from our laboratory where the loss of neurons found in the mPFC occurred more in females than males, producing a sex difference in the number of neurons in adulthood (Markham et al, 2007). These data are also similar to those

our laboratory has observed in the visual cortex. Much like the mPFC, there are sex differences in the rat visual cortex, with males having more neurons and a larger volume than females (Reid and Juraska, 1992; Nunez et al, 2002). In the visual cortex, it was found that removing the ovaries before puberty attenuated the neuronal loss (Nunez et al, 2002). Additionally, the removal of the testes before puberty had no significant effect on neuron number, similar to the current study. However, unlike the mPFC, layers 2/3 of the visual cortex were sensitive to the loss of ovarian hormones.

It is likely that both neurons and glia in the mPFC of the intact female are being lost during puberty through estrogen induced apoptosis. In early rat brain development, estrogen, via aromatized testosterone, induces apoptosis in some brain regions while concurrently saving cells in other nuclei (Arai et al, 2003; Tsukahara, 2009). The mechanism behind the region specificity is not known, but it has been shown that estrogen causes cell death by acting on cellular proteins in the apoptotic pathway (for review see Tsukahara, 2009; McCarthy, 2008) and can be blocked by an L-type voltage-gated channel blocker, confirming excessive calcium-induced cell death (Nunez and McCarthy, 2003; 2004). How estrogen is interacting with these proteins is also not known, but with the use of knockouts it has been shown that both estrogen receptor (ER)  $\alpha$  and ER $\beta$  are both required for the sex differences occur in the AVPV (Bodo et al, 2006). Both receptor types have been found in the adult cortex (Shughrue et al, 1997; Shughrue and Merchenthaler, 2001; Simerly et al, 1990) and during development estrogen binding remains high until P25 (Shughrue et al, 1990) then rises again in late adolescence at P50 (Presl et al, 1971). Whether or not these changes in estrogen binding relate to alterations in estrogen receptor levels or if they are involved in neuroanatomical changes during adolescence has not been established. Moreover, naturally occurring cell death has not been studied in adolescence, but

our laboratory has demonstrated a wave of cell death, just prior to puberty, at P25 in the visual cortex that only occurs in females (Nunez et al, 2001). Alternatively, other possible ovarian steroids may also be the cause of cell death. To date, the role of progesterone, alone or in concert with estrogen, has not been examined in cell death. Furthermore, unlike neurons, glia continue to proliferate at high rates in the cortex throughout the adolescent period (Lee et al, 2000; Ligon et al, 2006; Cameron and Dayer, 2008; Ahmed et al, 2008; Koss and Juraska, unpublished data). Therefore, we cannot discount the idea that ovarian hormones could be both inducing cell death in neurons and attenuating the proliferation of glia during puberty.

There were no effects of pubertal hormones on the number of neurons or glia in layers 2/3. Our laboratory's previous data did find significant losses of neurons in layers 2/3 but it appeared in both sexes, whereas the loss in layers 5/6 was only in females (Markham et al, 2007). Taken together, these data suggest the loss of cells in layers 5/6 during adolescence is dependent on ovarian hormones but the cell loss in layers 2/3 are hormone independent and must be due to a different mechanism. Other neuroanatomical measures such as dendritic modifications follow a similar pattern with more dendritic pruning occurring of layer 5 neurons in females from P35 to P90 (Koss, chapter 3), while the growth in dendrites of layer 3 occurs in both sexes from P30 to P90 (Markham et al, 2012). Dendritic spine density does not show the same lamina specificity to pubertal hormones. Measurements of basilar dendritic spines have shown to decrease in layer 5 of both sexes (Koss, chapter 3), whereas only females lose spines in layer 3 (Markham et al, 2012). Interestingly, some studies have identified that more ER $\beta$  is localized in layer 5 than layers 2 or 3 (Shughrue and Mechenthaler, 2001). A better understanding of the mechanism by which the pubertal hormones affects pruning during adolescence may help explain the lamina specificity.



The functional implications of this hormone dependent development are currently unknown. As shown in chapter 2, there have been no reported sex differences in PFC-dependent behavior (Koss et al, 2011). However, sex differences in activity and maze learning do not appear until after puberty (Kanit et al, 2000; Krasnoff and Weston, 1976), and no study has compared the cognitive behavior of rats who were gonadectomized before puberty. More research is needed to test the functional differences between males and females in the mPFC. Moreover, there are many other alterations occurring in the adolescent mPFC simultaneously such as alterations in monoamine, glutamatergic, and GABAergic neurotransmitter receptor levels (for review see Spear, 2000) that could also be changing behavioral phenotypes. Whether the ovarian steroids during adolescence are also affecting cellular characteristics other than the number of neurons in the mPFC remains to be elucidated.

## Tables

Table 5.1 Body and Brain Weights

	<b>Male Sham</b>	<b>Male GDX</b>	<b>Female Sham</b>	<b>Females GDX</b>
<b>Body Weight (g)</b>	<b>456.2 ± 10.9</b>	<b>374.6 ± 7.5*</b>	<b>251.9 ± 4.8</b>	<b>344.3 ± 6.1*</b>
<b>Brain Weight (g)</b>	<b>1.90 ± 0.03</b>	<b>1.91 ± 0.02</b>	<b>1.74 ± 0.03</b>	<b>1.86 ± 0.03*</b>

\*significantly different from same sex sham

## Figures

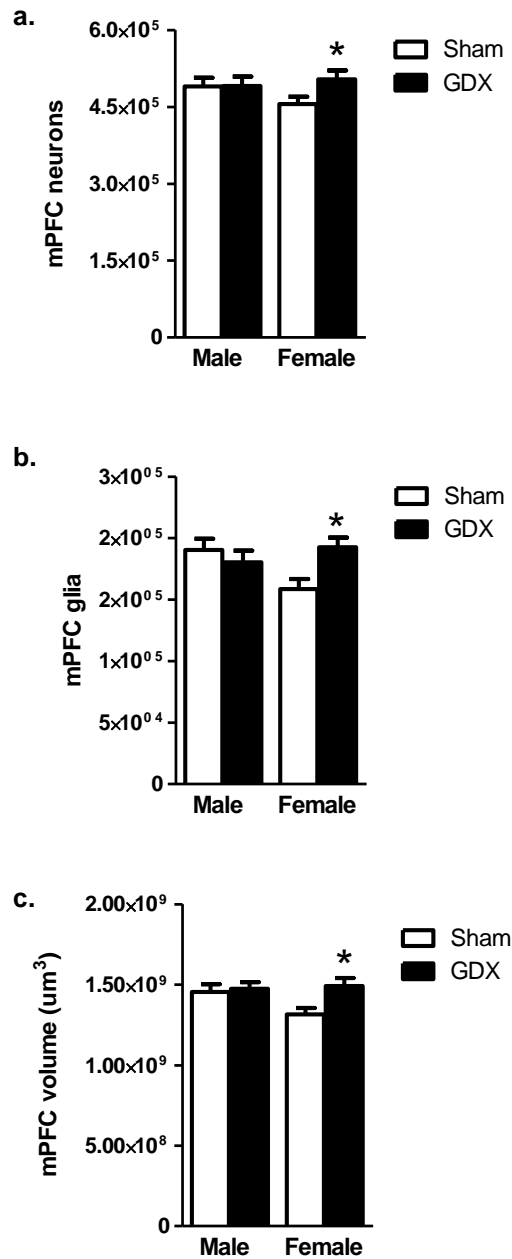


Figure 5.1 Measures of the entire mPFC (all layers combined). Significant differences were found between female gonadectomized (GDX) and female sham animals in neurons (a), glia (b) and volume (c). GDX males were no different than sham males in any measure. \*significantly different from same-sex sham group

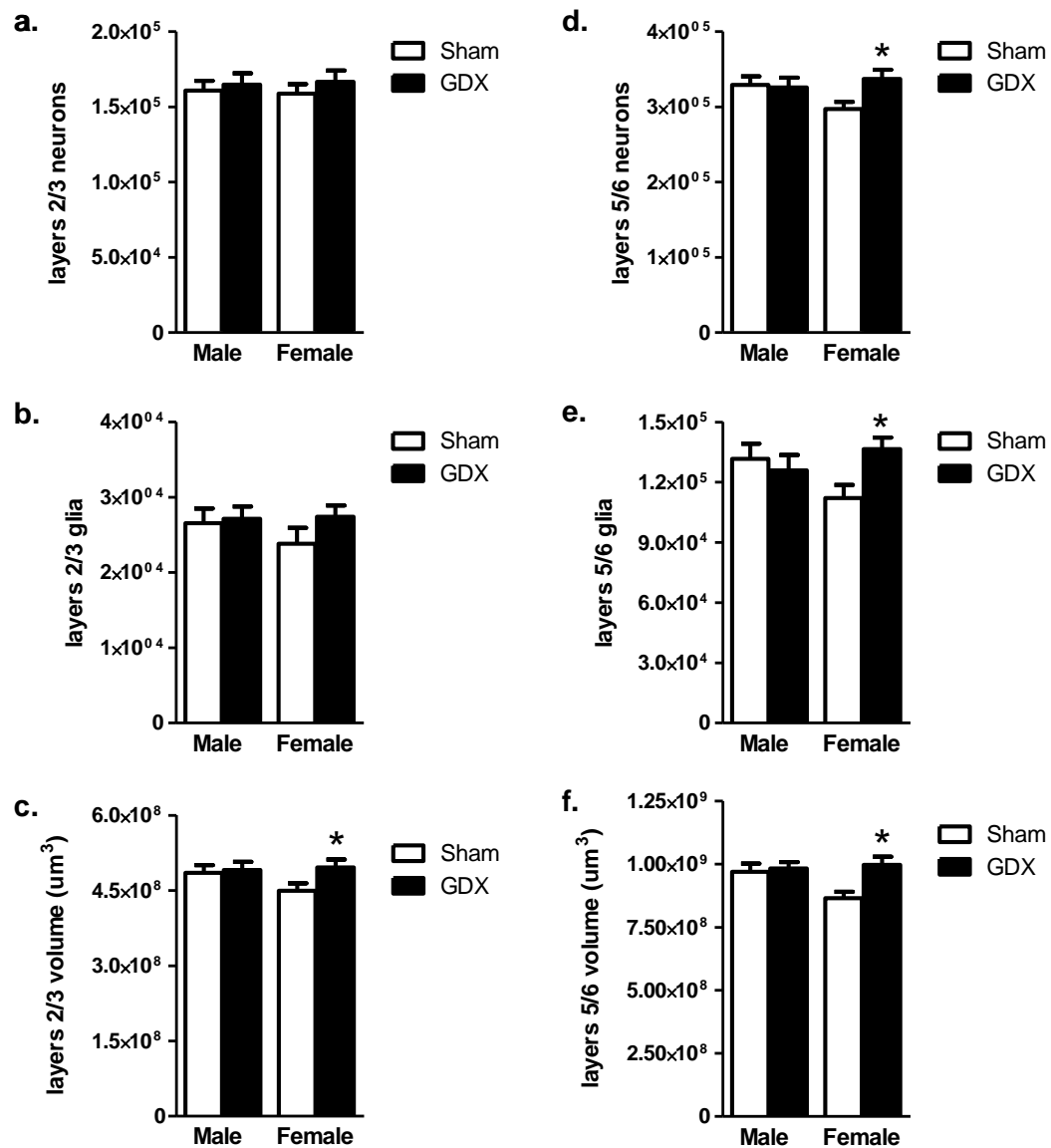


Figure 5.2 Measures by layer in the mPFC. Layers 2/3 (a-c) showed no changes in neurons or glia after (GDX) in either sex but female GDX animals did have significantly larger mPFC volume. In layers 5/6 (d-f) all measures were significantly different between the female GDX and female sham animals indicating OVX females had more neurons and glia as well as a larger volume. GDX males were not significantly different when compared to their intact controls.

\*significantly different from same-sex sham

## **Chapter 6**

## **Conclusions**

The set of studies described here focused on the adolescent development of the medial prefrontal cortex (mPFC) and the basolateral amygdala (BLN). In chapter 1, we demonstrated deficits in adolescent PFC-dependent behavior that may be a consequence of the continuing development in the mPFC during adolescence. In chapter 3, our data adds to the prior work from our laboratory showing significant dendritic alterations in both structures during the juvenile and adolescent period. Two additional studies were conducted to examine how extrinsic and intrinsic factors may affect the loss of neurons and glia previously reported in our laboratory. Since alcohol is readily consumed by human adolescents it may impact the development occurring during this time and thus permanently altering the number of neurons and glia in adulthood. In this study we found that the normal loss of neurons was not augmented by alcohol. However, a decrease in glia was found in the male mPFC only, with no effect in the BLN. The functional consequences of the loss of glia are not known, but could contribute to decreased synapse function or myelination depending on the type of glia that is lost. Next, we explored the effects of pubertal hormones. Since pubertal hormones have been shown to trigger cell death in development it was logical to assume that these hormones may be initiating the cell loss seen in adolescence. We examined the number of neurons and glia in the mPFC after eliminating pubertal hormones by performing gonadectomies prior to puberty. Results indicated that ovariectomy to females eliminated the cellular loss in females, but castration to males had no effect. These studies build on previous research from our laboratory to further obtain a full perspective of structural changes and intrinsic and extrinsic factors that may perturb development during the adolescent period.

## **Extending Conclusions of Human MRI Adolescent Studies through Animal Models**

Imaging studies in humans have documented increases in gray matter volume throughout childhood followed by a decrease in adolescence within the PFC (e.g. Giedd et al, 1996b). This highlights the late development of the PFC. The mechanism behind this development is still not fully known, but the current and prior studies using the rat as a model have provided some answers. Data has indicated that increases in dendritic arborization and spines are likely responsible for the pre-adolescent increase in volume whereas a balance of neuron loss, dendritic spine and synapse loss, as well as lamina-specific dendritic retractions and growth contribute to the decreases in volume during adolescence (Markham et al, 2007; 2012; Koss et al, chapter 3). This overproduction of synapses and dendritic material followed by pruning is a hallmark of early neural development within the sensory areas of the brain, and thus further support the viewpoint of late development within the mPFC.

Late neuroanatomical development has also been shown in the BLN. Much like the mPFC, the BLN increases in dendritic branching and dendritic spines between P20-P35 and neurons decrease during adolescence as well. In contrast, the BLN dendrites are increasing in the number of dendritic branches with little overall increase of the dendritic tree and no pruning in dendrites or spines. Unfortunately, this does not mimic human MRI studies that have reported an increase in gray matter volume in the amygdala in childhood and throughout adolescence. This is likely due to lack of the regional specificity in MRI studies. The current study and our prior studies have specifically researched the BLN, whereas MRI studies observe changes in the whole amygdala. This means that the results from MRI studies report the summation of adolescent development within all the nuclei, which could be very different. For instance, the medial amygdala has been shown to increase in volume throughout adolescence (Romeo and

Sisk, 2001). Therefore, more research is needed to validate the use of the rat in the study of adolescent development in the amygdala.

### **Sex Differences and the Impact of Pubertal Hormones in Adolescent Neural Development**

In chapter 5, it was shown that ovarian hormones, not androgens, have a significant role in both neuronal and glia loss found in females during the adolescent period. However, this was limited to layers 5/6 of the mPFC. Similarly, prior research in our laboratory found that females during adolescence lose neurons within layers 5/6, but males do not, while both sexes lose neurons in layers 2/3. The ovarian hormone, estrogen, has been found to be a mediator of apoptosis during early development by regulating the balance of pro-apoptotic and anti-apoptotic proteins of the BCL2 family. Interestingly, ER $\beta$  receptors in the cortex are particularly localized in the deeper layers of the cortex (Shughrue et al, 2001), may be making these layers more sensitive to estrogen than layers 2/3. More studies are needed to clearly confirm the role of estrogen and ER $\beta$  receptors in the cellular losses of layers 5/6. In layers 2/3 both sexes lose neurons and this loss was not sensitive to ovarian or testicular hormones. The exact mechanism behind this cellular loss in both sexes in layers 2/3 is unknown. It is most likely due to other adolescent changes in the cellular environment that influence the apoptotic pathway. Since the BLN found similar cell loss in males and females we also believe that the mechanism underlying the cell loss is also not due to pubertal hormones. However, further research is needed to substantiate this hypothesis.

Sex differences were also observed in the retractions of the basilar dendrites of the mPFC. Pubertal hormones and their role in dendritic branching have not been fully studied, especially during development in the cortex. However, the sex differences make it is easy to



predict that pubertal hormones, specifically ovarian hormones, play a role. In the medial amygdala, retractions of dendrites during adolescence have been found to be due to androgens (Zehr et al, 2006), and also in early development estrogen modulates growth of neurites, spines and synapses (for review see McCarthy, 2008). Even though in our study we found no sex differences in spine losses in the mPFC, it is still possible that some of the loss is due to gonadal hormones. Similar evidence has been found in the adolescent hippocampus where both males and females lose spines over the adolescent period, but prepubertal castration of males, not ovariectomy of females, attenuates the loss (Meyer et al, 1978; Yildrin et al, 2008). Alternatively, since dendritic retractions and spine losses both infer synapse loss throughout the adolescent period, there is a possibility that these losses could just be an effect of the cellular loss in this area. However, if true, this should have occurred in the BLN. Additionally, other changes during adolescence may influence dendritic arborization and spines, such as the NMDA receptor and many other excitatory neurotransmitter receptors which are pruning simultaneously (Gould et al, 1991; Kalsbeek et al, 1988; Andersen et al, 2000; Insel et al, 1990; Kellogg et al, 1993; Primus and Kellogg, 1991). Understanding the specific mechanisms, both gonadal steroid dependent and not, of neuroanatomical changes that we have established is clearly the next step in this line of research.

Unfortunately, we found no evidence of sex differences in adults or adolescents within the PFC-dependent task used in chapter 1. This result was unexpected, since there were significant sex differences in the number of neurons in the adult mPFC, as well as sex differences in the loss of neurons and dendritic pruning across the adolescence period (Markham et al, 2007; Koss et al, chapter 3). In the non-delayed portion of the test adult females outperformed adult males, and when comparing adolescents, females had significantly more

perseverative errors, which did not occur in the delayed portion of the task. It should be noted that the non-delayed portion of this task was done prior to puberty in males (~P42) and just before or right at puberty in females (~P35), unlike the delayed portion which occurred after puberty in both sexes. Moreover, the non-delayed portion of the task is known to be more associated with hippocampal functioning than mPFC (Lalonde, 2002). So conclusions from these findings are somewhat unclear. Still others have found sex differences in PFC-dependent behaviors in the adult and adolescent period. Female adolescents have slower behavioral extinction in both aversive and non-aversive conditioning tasks (Baran et al, 2009; Hammerslag et al, in press) and female adolescents demonstrated incomplete extinction which was not present in adolescent males (Hammerslag et al, in press). Given these findings it is possible that sex differences are task-dependent. Much more research is needed in order to fully confirm behavioral sex differences, in adolescents and adults, and how they correlate to the sex differences found in our neuroanatomical findings.

On a cautionary note, all of the neuroanatomical measures performed in our laboratory have compared the specific ages of P20, P35 and P90. This is design of experiment is only providing a snapshot of what is occurring during the adolescent period. Therefore, it is possible we could be missing important neuroanatomical changes that are occurring during other ages. Adding to this, the sex differences found within our measures may not be truly accurate for these reasons. Instead of sex differences in the changes, it is possible that there are sex differences in the timing of the development that correlates with the puberty. As mentioned, the average age of puberty in females is P35 but the average age of puberty in males is later at age P42 (Koss et al, chapter 5) which may mean that the changes are happening past P35 which may be unseen by our experiments. Future research should include a time point closer to male puberty, as well as

P35, to ensure we do not miss the effects of puberty onset in the males. On the other hand, since castrations had no effect on the cellular changes in males over the adolescent period, it is probable that no further changes in neuron or cell death will occur, but because it is unknown how pubertal hormones are affecting dendrites, dendritic changes may still be still be happening in males that are not revealed in these studies.

To conclude, it should be noted that the investigation of adolescent development is rarely studied in both sexes, even though puberty is a hallmark of adolescence. For example the majority of the research on neurotransmitter receptor changes has only been looked at males, even though it is clear that ovarian hormones have effects on all neurotransmitter systems in adulthood (Renner and Luine, 1986; Fink et al, 1996; Biegon et al, 1983; Majewska, 1992; Herbison, 1997). Moreover, since we are providing some evidence for sex differences in the amount of synapse loss in adolescence it is likely that there are similar changes in neurotransmitter innervation. There is one study that has shown males to have a greater overproduction of D1 and D2 receptors in the nucleus accumbens and striatum during adolescence. The study goes on to show males having greater elimination in the striatum than females, but similar pruning in the nucleus accumbens (Andersen et al, 1997). It would be interesting to investigate if this pattern remains constant in other regions of the brain, such as the mPFC.

### **Possible Outcomes of Abnormal Adolescent Development**

Similar to early development the adolescent period has proven to be a critical period of development where organizational changes occur. Whether this period is an extension of development or a novel period of time that is sensitive to specific changes is unknown.

Currently there is evidence for both. As mentioned, the medial amygdala increases in cells and volume during adolescence in the males (Romeo and Sisk, 2001; Ahmed et al, 2008). This has been shown to be organized by androgens within the adolescent period (Ahmed et al, 2008; Schulz et al, 2009). Furthermore, it has been demonstrated that this structure is sensitive to androgens from the early postnatal development period until the end of adolescence. This suggests that the adolescent period is an extension of early development (Schulz et al, 2009). In contrast, work in the AVPV, which is bigger in females, has found that cell death in males occurs in a precise window from P7-P10 and does not persist after P10 (Arai et al, 1996). More recently, work has shown that the AVPV of females, not males, continues to add cells during adolescence to augment the sexual dimorphism in adulthood (Ahmed et al, 2008). This work implies that adolescence is a separate and novel period of development. Given these lines of limited evidence, it is possible that some brain regions are extensions of early development and some are not. It would be very interesting to test when the mPFC becomes sensitive to ovarian hormones, when the window closes and what specific mechanisms creates these specific windows of development. Either way the changes in adolescence are permanent which creates a period of vulnerability where abnormal development, either from extrinsic or intrinsic factors, during this time may be detrimental to cognitive and emotional outcomes.

In chapter 4, we observed a decrease of glia number in the male mPFC due to adolescent alcohol exposure, but no changes in females or in the number of neurons. This demonstrates a possible neuroprotective role of ovarian hormones in the mPFC which seems contrary to the pro-apoptotic role of ovarian hormones concluded in chapter 5. However, since the effect was only observed in glia, not neurons, it could be that alcohol is attenuating glia proliferation not cell death. Evidence for this is supported by our laboratory's previous data showing the number of

glia increase over the adolescent period in males but decrease in females (Markham et al, 2007), and thus establishing a basis for the sex difference in the effects of alcohol. Other illicit drugs may have other detrimental effects when used or abused during the adolescent period. For instance, most drugs of abuse affect the dopaminergic system, which is continuing to develop during adolescence particularly in the mPFC (Tseng and O'Donnell, 2005; 2007a; 2007b).

Other work has suggested that the adolescent brain may be particularly sensitive to the development of mental disorders. This could be due to the continuing development within specific brain regions involved in these illnesses. For instance, the lack of pruning of, or the abundance of, synapses and neurons in the PFC has been associated with autism and mental disorders (Kulkarni and Firestein, 2012; Courchesne et al, 2011). Since we and others have shown that the mPFC continues to prune the dendritic tree, spines and neurons throughout adolescence (Markham et al, 2007; Koss et al, chapter 3) which could be altered by intrinsic or extrinsic factors to increase the susceptibility to certain mental disorders. Moreover, stress has been shown to be a factor that alters dendrites in the mPFC during early development (Chocyk et al, 2013; Muhammad et al, 2012) and adulthood (Brown et al, 2005). In addition, sex differences have been found in the response to stress in the hippocampal dendrites during adulthood (Shors et al, 2001). Dendritic alterations to stress or any other factor have not been investigated in adolescent development but with the evidence from early development and adulthood, it is difficult to conclude there would be no permanent changes that may lead to dysfunctional behaviors. Furthermore, through electrophysiological studies it has been found that neurons in the mPFC do not yet have adult responses to dopaminergic innervation until after the adolescent period (Tseng and O'Donnell, 2005; 2007a; 2007b), providing a possible functional consequence for neural alterations which could be permanently altered by excessive pruning or lack of

pruning occurring in the adolescent period. A direct relationship between abnormal adolescent development and mental illness and cognitive deficits still needs to be established, but the work presented here, along with other research, may create a basis on which to build.

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